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Host Responses to Human Neural Cell Therapy in Spinal Cord Injury

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Cover illustration:
Top left panel: Human fetal neural stem/progenitor cells (hfNPCs) in culture as a neurosphere. Neural progenitor cells are Nestin immunoreactive (green) and nuclei are labeled with Hoechst (blue).

Top right panel: A neurosphere with hfNPCs undergoing initial differentiation. Nestin (green) and glial fibrillary acidic protein (GFAP, red) and nuclei are labeled with Hoechst (blue).

Bottom left panel: hfNPCs under differentiation expressing nestin (green) and GFAP (red) and nuclei are labeled with Hoechst (blue).

Bottom right panel: hfNPCs under differentiation expressing β tubulin-III (green) and nuclei are labeled with Hoechst (blue).

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人生重要的不是所站的位置，而是所朝的方向。

The important thing in life is not where you stand, but rather where you are heading.

Chinese saying

To my parents with love

ABSTRACT

Spinal cord injury (SCI) is a devastating condition without a cure. The SCI process comprises an initially mechanical trauma and a secondary cascade of events including a robust inflammatory and immune response. Experimental neural cell therapy in animal models have presented a series of beneficial effects such as neuroprotection, cell replacement, remyelination and axon regeneration. However, it is still to a large degree unclear how the host immune and nervous system respond to and interact with human donor neural stem/progenitor cells (NPCs). Therefore, features of human NPCs and their effects on potential host responder cells such as lymphocytes, microglia and spinal cord neural cells were studied *in vitro* and *in vivo*.

Features of two relevant types of donor human NPCs: human embryonic stem cell-derived NPCs (hESC-NPCs) and human fetal spinal cord-derived NPCs (hfNPCs), cultured under equivalent conditions were evaluated. hESC-NPCs and hfNPCs presented relatively similar expression patterns of human leukocyte antigen, co-stimulatory and adhesion molecules and were mostly not affected by two inflammatory cytokines of interest in SCI. Unstimulated hfNPCs secreted more transforming growth factor-β (TGF-β) but similar level of interleukin (IL)-10 compared to hESC-NPCs. In contrast to hfNPCs, hESC-NPCs showed increased release of TGF-β and IL-10 under *in vitro* conditions mimicking inflammation. Both human NPCs reduced an alloreaction between non-compatible allogeneic peripheral blood mononuclear cells (PBMCs) and up-regulated $CD4^{+}CD25^{+}$forkhead box $P3^{+}$ T cells, identified as induced regulatory T cells. However, hESC-NPCs but not hfNPCs dose-dependently triggered allogeneic PBMC proliferation, which may be at least partly due to TGF-β signaling. To conclude, differences in immunocompetence and interaction with allogeneic PBMCs were observed between hESC-NPCs and hfNPCs. These differences may be crucial for the host response in neural cell therapy.

To study the interaction between human NPCs and allogeneic microglia, an *in vitro* co-culture model was employed. The presence of microglia enhanced the survival and proliferation of hfNPCs, but hindered their differentiation. In the presence of hfNPCs, the survival, proliferation and phagocytosis of human microglia was increased. The expression of the neuroimmune regulatory protein CD200 on hfNPCs, and the CD200 receptor on microglia, were enhanced in co-cultures, accompanied by increased secretion of TGF-β, indicating an anti-inflammatory feature of the co-cultures. To conclude, in this model of the naïve encounter of human donor NPCs with host microglial cells, the interplay between human NPCs and allogeneic microglia significantly affected their respective proliferation and phenotype. The neural cell and microglial interaction presented features that may benefit host neuroprotection and repair.

To further study host responses in neural cell therapy, hfNPCs were xenotransplanted to rats with severe or moderate SCI with or without immunosuppression. hfNPCs, expanded *in vitro* for 5 passages (NPC-P5) and grafted acutely to severe SCI rats, were completely rejected. In acute transplantation of NPC-P0 and delayed transplantation of NPC-P5 to rats with moderate SCI, a complete rejection occurred in 40 and 33%, respectively. Locomotor function was not significantly different between groups, indicating that neither transplantation nor rejection altered functional outcome during the 6-week long study. Host microglial activation at the SCI epicenter was reduced in hfNPC transplantation groups compared to lesion alone in both a xeno- and allotransplantation model. In conclusion, human neural transplantation may result in a host rejection but still reduce the microglial response at the SCI epicenter.

Finally, the injured spinal cord response to human NPCs was evaluated in two different rat SCI models and a human allograft *in vitro* model. Spinal cord-derived hfNPCs (SC-NPCs) transplanted subacutely after contusion injury improved host locomotor function. In a compression SCI model, acutely or subacutely grafted SC-NPCs, but not chronically transplanted SC-NPCs or subacutely grafted forebrain-derived hfNPCs, enhanced functional recovery. Four months after transplantation, the number of surviving host spinal cord neurons was highest in acutely and subacutely transplanted groups, accompanied by the best hindlimb function. This suggests that transplanted SC-NPCs improve functional recovery by a neuroprotective effect. In addition, grafted SC-NPCs reduced the percentage of injury-induced apoptotic cells in a human organotypic spinal cord culture system.

In summary, human NPCs exert immunomodulatory and neuroprotective effects in SCI models. The human NPC origin, the host injury severity and the time point of neural cell therapy in SCI may affect the host–donor interaction and host response. With increased knowledge and awareness of these factors, human neural cell therapies for SCI can be improved to achieve higher therapeutic efficacy.

LIST OF PUBLICATIONS

I. **Jia Liu**, Cecilia Götherstrom, Magda Forsberg, Eva-Britt Samuelsson, Jiang Wu, Cinzia Calzarossa, Outi Hovatta, Erik Sundström, Elisabet Åkesson. ***Human neural stem/progenitor cells derived from embryonic stem cells and fetal nervous system present differences in immunogenicity and immunomodulatory potentials in vitro.*** Stem Cell Research. 10(3):325-337; 2013.

II. ***Jia Liu**, *Erik Hjorth, Mingqin Zhu, Cinzia Calzarossa, Eva-Britt Samuelsson, Marianne Schultzberg Marianne and Åkesson Elisabet. ***Interplay between human microglia and neural stem/progenitor cells in an allogeneic co-culture model.*** Journal of Cellular and Molecular Medicine. *In press,* 2013. doi: 10.1111/jcmm.12123

III. ***Jia Liu**, *Jenny Odeberg, Cinzia Calzarossa, Jinghua Piao, Madeleine Cederarv, Nina Wolmer-Solberg, Cecilia Götherström, Lena Holmberg, Eva-Britt Samuelsson and Elisabet Åkesson. ***Host responses after human neural xeno- and allotransplantation to the injured spinal cord.*** Submitted Manuscript.

IV. *Mia Emgård, *Jinghua Piao, Helena Aineskog, **Jia Liu**, Cinzia Calzarossa, Jenny Odeberg, Lena Holmberg, Eva-Britt Samuelsson, Bartosz Bezubik, Per Henrik Vincent, Scott Falci, Åke Seiger, [§]Elisabet Åkesson and [§]Erik Sundström. ***Neuroprotective effects of human spinal cord-derived neural precursor cells after xeno- and allotransplantation to the injured spinal cord.*** Submitted Manuscript.

*, [§] These authors contributed equally to the study.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

7-AAD	7-amino-actinomycin D
ASIA	American Spinal Injury Association
BDNF	Brain derived growth factor
BMSC	Bone marrow stromal cells
CFSE	carboxyfluorescein diacetate succinimidyl ester
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CSPG	Chondroitin sulphate proteoglycans
EAE	Experimental autoimmune encephalomyelitis
ESC	Embryonic stem cells
FBr-NPC	Sub-cortical forebrain-derived hfNPCs
FGF	Fibroblast growth factor
FOXP3	Forkhead box P3
GDNF	Glial cell-derived growth factor
GFAP	Glial fibrillary acidic protein
GRP	Glial restricted precursors/progenitors
hESC-NPC	Human NPCs derived from ESCs
hfNPC	Human NPCs derived from first trimester nervous system
HLA	Human leukocyte antigen
IFN-γ	Interferon-γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent stem cells
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NIReg	Neuroimmune regulatory proteins
NPC	Neural stem/progenitor cells
NT	Neurotrophin
OEC	Olfactory ensheathing cells
OPC	Oligodendrocyte progenitor cells
P	Passage
PBMC	Peripheral blood mononuclear cells
PSA-NCAM	Polysialylated-neural cell adhesion molecule
SAP102	Synapse-associated protein 102
SC-NPC	Spinal cord-derived hfNPCs
SCI	Spinal cord injury
TGF-β	Transforming growth factor-β
Th	T helper cells
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cells

1 INTRODUCTION

1.1 Spinal cord injury (SCI) – an overview

Spinal cord injury (SCI), due to sudden and/or sustained trauma, is a devastating condition including functional deficits as well as emotional, social, and financial burdens. Physical disruption of spinal cord circuitries, descending and ascending axons results in motor and sensory dysfunction, often including spasticity, neuropathic pain, paresthesia and autonomic dysreflexia. Furthermore, SCI also leads to an increased risk of cardiovascular complications, osteoporosis, pressure ulcers, and urinary tract infections (Hagen *et al.* 2011).

Worldwide, the reported annual incidences of SCI range from 2.3 in Canada to 83 in Alaska per million inhabitants (Hagen *et al.* 2012). Two recent Scandinavian studies have revealed trends of increased incidence rates over the past few decades (Ahoniemi *et al.* 2008; Hagen *et al.* 2010). SCI victims are predominantly male (80.7%) and nearly half are injured between the ages of 16 and 30 (National Spinal Cord Injury Statistical Center, USA, 2012; Levi *et al.* 1995). In USA, the most common causes of traumatic SCI are traffic accidents, falls, violence (primarily gun shot wounds) followed by sports-related injuries (National Spinal Cord Injury Statistical Center, USA, 2012). In Europe, violence causes a smaller proportion of the injuries compared to the USA. Cervical SCIs account for up to 75% of the total number of SCIs, followed by thoracic and lumbar SCIs (Devivo 2012; Hagen *et al.* 2012).

The SCI process includes two sequential phases. First, the initially mechanical trauma that causes an immediate structural and physiological disruption of axons, neural cell damage and vascular disruption at the site of injury within seconds (Hulsebosch 2002). Microvascular tearing leads to hemorrhage, edema, ischemia, substantially reducing tissue perfusion and delivery of oxygen and nutrition to tissues, all of which further exacerbate the neural injury (Tator and Fehlings 1991). A secondary degenerative phase is initiated by a cascade of vascular, biochemical and cellular processes that can last from days to years (Sekhon and Fehlings 2001). The ischemia and edema at the injury epicenter develop and spread to the adjacent tissues within 24-48 hours after the initial trauma (Tator and Fehlings 1991). The hemorrhage and microvascular reperfusion increase the level of free radicals, contributing to a progressive oxidation of fatty acids in cellular membranes (named lipid peroxidation). The oxidative stress

disables key mitochondrial respiratory chain enzymes, alters DNA and associated proteins and inhibits sodium-potassium ATPase, inducing metabolic collapse and further cell death by necrosis and/or apoptosis (Lewen *et al.* 2000). Compromised membrane integrity and calcium transporters rapidly lead to increased intracellular calcium concentrations. Extracellular accumulation of excitatory neurotransmitters such as glutamate occurs rapidly after injury in response to ischemia and malfunctional cellular transporters. Activation of ionotropic glutamate receptors increases intracellular accumulation of calcium further, which triggers a multitude of events that also end in a progressive cell death and tissue damage (Park *et al.* 2004). The loss of oligodendrocytes, which are particularly vulnerable to excitotoxicity, results in demyelination of axons and conduction deficits (Hulsebosch 2002). The breakdown of the blood-brain barrier facilitates the infiltration of peripheral immune cells (e.g. neutrophils, monocytes, macrophages and lymphocytes). The infiltration of immune cells initiates the inflammatory response in the injured spinal cord, accompanied by the intraspinal microglial activation and the production of pro-inflammatory cytokines (Trivedi *et al.* 2006). In addition, reactive astrocytes around the lesion together with invading meningeal fibroblasts, vascular endothelial cells, microglia, oligodendrocyte precursors and myelin debris form a non-permissive glial scar, sealing the lesion from the intact spinal cord (Fawcett and Asher 1999; Fitch *et al.* 1999). Recently, Göritz *et al.* also demonstrated that pericytes after SCI gave rise to stromal cells that even outnumbered the astrocytes in the scar (Goritz *et al.* 2011). A scar in the injured spinal cord hinders re-growth and repair of injured axons by production of inhibitory molecules such as chondroitin sulphate proteoglycans (CSPGs); on the other hand, it can also re-establish the physical and chemical integrity of the injured spinal cord and minimize the spread of cellular damage (Rolls *et al.* 2009; Silver and Miller 2004).

1.2 Inflammation and immune responses after SCI

SCI initiates robust inflammatory and immune responses, which persists for several weeks to months after the lesion. The response is characterized by infiltration of peripherally derived immune cells (such as neutrophils, monocytes, macrophages and lymphocytes), activation of glial cells (microglia and astrocytes) within the spinal cord itself, and the release of cytokines (Beck *et al.* 2010; Fleming *et al.* 2006; Popovich *et al.* 1997). The inflammatory and immune reaction in SCI not only contributes to exacerbate the injury and damage healthy tissues, but is also a critical host defense mechanism to eliminate invading pathogens and to clear debris.

Neutrophils in SCI

Neutrophils are the first peripheral immune cells to arrive at the injury site after SCI, reaching a peak at three days post-injury but with an additional second peak several weeks later (Carlson *et al.* 1998; Fleming *et al.* 2006; Kigerl *et al.* 2006). They are particularly abundant around the central gray matter hemorrhagic and necrotic areas (Fleming *et al.* 2006). The recruitment of neutrophils from peripheral blood may be enhanced by adhesion molecule expression (e.g. transmembrane intracellular adhesion molecule and platelet–endothelial cell adhesion molecule) on the cellular membrane to guide them to the injury site. Neutrophils are involved in the phagocytosis of tissue debris and the modulation of the inflammatory response by releasing proteases (e.g. matrix metalloproteinases (MMPs) and elastase), free radicals and cytokines (Trivedi *et al.* 2006). For example, MMP-9 is reported to cleave myelin basic protein (MBP) and contributes to demyelination of healthy axons (Noble *et al.* 2002), early secondary tissue damage and hemorrhagic injury (Fleming *et al.* 2006). However, neutrophils do not only have detrimental bystander effects. A recent study showed that depleting neutrophils by selective antibody treatment in SCI mice resulted in a marked increase in tissue damage and impaired functional recovery, confirming also the protective and regulatory role of neutrophils in SCI (Stirling *et al.* 2009).

Macrophages and microglia in SCI

Monocytes enter the injury site and quickly differentiate into macrophages (Blight 1985; Popovich *et al.* 1997). Resident microglia respond to an injury in the spinal cord rapidly, within minutes (Fleming *et al.* 2006). Recruited macrophages and activated microglia contribute to the secondary tissue damage and inflammatory response in part through the production of toxic mediators (e.g. tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6) and enzymes (e.g. inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)) (David and Kroner 2011; Jones *et al.* 2005; Pais *et al.* 2008). Upon exposure to necrotic neural cells, the up-regulation of major histocompatibility complex (MHC) class II and co-stimulatory molecules on macrophages and microglia enables them to present antigen to CD4+ T cells and to participate in the adaptive immune response (Pais et al. 2008). Activated macrophages and microglia are efficient phagocytes and are capable of clearing debris from the injured spinal cord (Smith et al. 1998). Cytokines (e.g. IL-1, TNF-α)

derived from activated microglia may also contribute to oligodendrocyte proliferation, remyelination, axon re-growth and revascularization at the injury site (Jones *et al.* 2005). Other soluble factors, such as transforming growth factor (TGF)-β, brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and neurotrophin (NT)-3, produced by microglia may help tissue repair by enhancing axonal sprouting, suppressing macrophage activation and blocking the production of reactive oxygen intermediates and inflammatory cytokines (Batchelor *et al.* 1999; David and Kroner 2011). Macrophages and microglia can also increase the uptake of extracellular glutamate through transporter proteins such as GLAST and GLT-1 (Gras *et al.* 2012; van Landeghem *et al.* 2001). Activated macrophages and microglia are highly plastic cells, and can play a neurotoxic and/or neuroprotective role (David and Kroner 2011). The neurotoxic activities are usually associated with the "classical activation (via Toll-like receptors or interferon (IFN)-γ)" M1 phenotype, and their release of pro-inflammatory cytokines, such as TNF-α and IL-1, contributing to neuronal dysfunction and cell death (Block and Hong 2005). While the protective activities are associated with the "alternative activation (by IL-4 or IL-13)" M2 phenotype, and the production of neuroprotective substances, such as anti-inflammatory cytokines and neurotrophic factors, promoting tissue repair by blocking the production of reactive oxygen intermediates and pro-inflammatory cytokines (David and Kroner 2011). Kigerl and colleagues reported the co-existence of M1 and M2 macrophages in the contused mice spinal cord, showing that M1 macrophage response was rapidly induced, neurotoxic and maintained at the injury sites, while the response of M2 macrophages was smaller, transient and promoted axon re-growth and limited the secondary inflammatory-mediated injury (Kigerl *et al.* 2009).

Lymphocytes in SCI

Lymphocytes are activated by neuroantigens that are released into the blood and lymphatic system (Ankeny and Popovich 2009). T cells progressively increase in number in parallel with the activation of microglia and influx of peripheral macrophages during the first week after SCI. The T cell number remains elevated for long (Ankeny and Popovich 2009; Sroga *et al.* 2003). Activated T cells contribute to microvascular injury, axonal impairment, neurotoxicity and regulation of macrophage functions (Jones *et al.* 2005). Chronically activated T cells participate in the pathological fibrosis and scarring (Wynn 2004). A subpopulation of T cells is activated by myelin protein from necrotic and apoptotic neural cells, called MBP-

reactive T cells. MBP-reactive T cells are reported to exacerbate axonal injury, demyelination and functional loss after experimental SCI (Jones *et al.* 2004). MBP-reactive T cells isolated from SCI rats induce transient paralysis and inflammation when injected to uninjured rats (Popovich *et al.* 1996). However, Schwartz and Kipnis proposed that trauma-induced activation of MBP-reactive T cells is a physiological rather than a pathological consequence of injury and should be boosted to achieve neuroprotection (Schwartz and Kipnis 2001). T cells can also produce neurotrophic factors and immunosuppressive cytokines to inhibit macrophage produced inflammatory cytokines (e.g. TNF-α) and promote the production of immunomodulatory cytokines such as TGF-β (Jones *et al.* 2005). SCI could also result in the activation of B cells and pathogenic autoantibody production within both cerebrospinal fluid and the spinal cord parenchyma (Ankeny *et al.* 2009). Injury-activated B cells could influence axonal regeneration and oligodendrocyte survival through the increased production of autoantibodies that are specific to myelin protein (Huang *et al.* 1999; Saltzman *et al.* 2013).

Reactive astrocytes in SCI

SCI triggers astrocytes to undergo phenotypic and morphological changes including hypertrophy, elevated proliferation and increased expression of intermediate filaments such as glial fibrillary acidic proteins (GFAP), nestin, and vimentin (Silver and Miller 2004). Reactive astrocytes could exert both detrimental and beneficial effects by releasing both pro- and anti-inflammatory cytokines such as TNF-α, IFN-γ, IL-1, IL-6 and TGF-β to regulate inflammation and secondary injury mechanisms (Karimi-Abdolrezaee and Billakanti 2012). Reactive astrocytes contribute to the production of inhibitory extracellular matrix components such as CSPGs, tenascins and collagen that obstruct axonal elongation and sprouting (Fitch *et al.* 1999; Shibuya *et al.* 2009). Reactive astrocytes play crucial roles in SCI repair with protective properties: reconstructing damaged blood-brain barrier, limiting infiltration of peripheral leukocytes and activation of resident microglia, modulating blood flow, up-take of excessive glutamate and producing antioxidants (i.e. glutathione) against oxidative stress (Karimi-Abdolrezaee and Billakanti 2012; Leal-Filho 2011). Reactive astrocytes can potentially promote tissue repair and regeneration by up-regulation of fibroblast growth factor (FGF)-2 and S100β, which are exclusive to reactive astrocytes but not activated macrophages and microglia, in the injured spinal cord (do Carmo Cunha *et al.* 2007). Ablation of reactive astrocytes in SCI animals caused

substantial vasogenic edema and widespread inflammation and tissue degeneration (Faulkner *et al.* 2004).

Complement system in SCI

The complement system plays a crucial role in the recruitment of mononuclear cells and macrophages (C3a and C5a) and in the deposition of the cytotoxic pore-forming membrane attack complexes (C5–C9) on the cell surface (David *et al.* 2012). Complement proteins are elevated in SCI patients (Rebhun *et al.* 1991). SCI causes the locally increased synthesis of complement proteins, which deposit on neurons, axons and oligodendrocytes (Anderson *et al.* 2004). Galvan *et al*. reported that the activation of the classical complement pathway via C1q is detrimental for tissue damage and functional recovery after SCI (Galvan *et al.* 2008). Mice deficient in C3 showed significant enhancement in functional recovery, tissue sparing and less necrosis, demyelination and neutrophil infiltration after SCI (Qiao *et al.* 2006). These results indicate that the complement contributes to secondary damage after SCI.

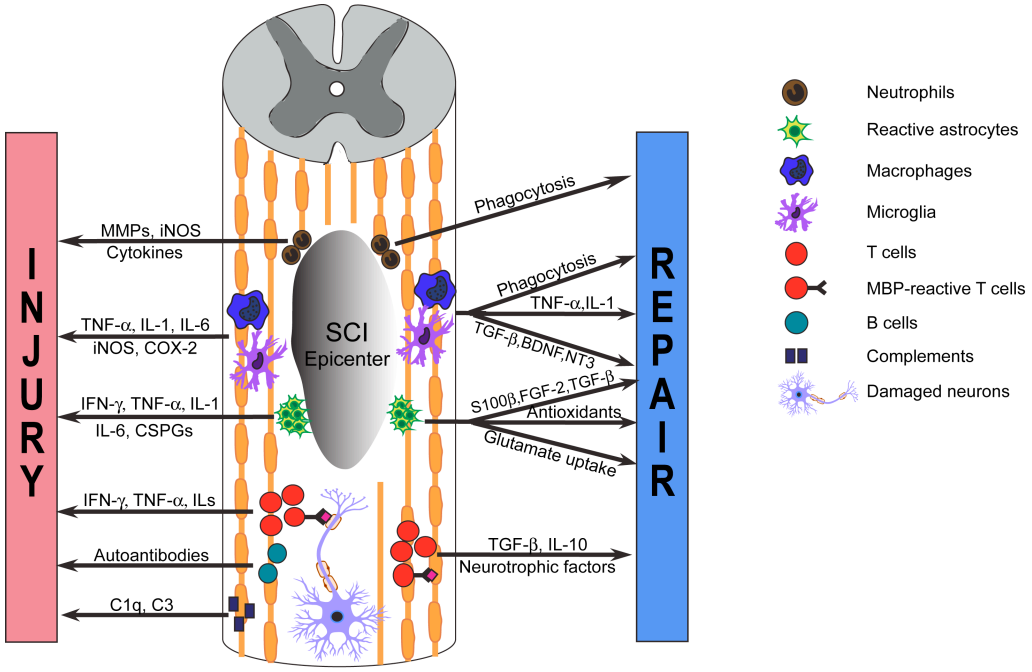

Fig.1. Schematic figure showing the role of the main immune cells and mediators involved in the inflammatory and immune response after SCI.

1.3 Treatment strategies for SCI

Below, some important and promising treatment strategies for SCI will be mentioned without the possibility to make the list complete within the frame of this thesis summary.

General management

Traumatic SCI usually includes fractures and ligament ruptures of the vertebral column, as a result, minor movements or manipulations of the unstable columnar region in the neck or back can worsen an already existing injury in the spinal cord. Thus, the first important pre-hospital action is immobilization of the patient using a cervical collar, head immobilization, and a spinal board (Ahn *et al.* 2011). Transportation of patients with acute SCI to hospital should occur without delay, and early transfer is preferable as it decreases overall mortality and complications (Ahn *et al.* 2011; Divanoglou *et al.* 2010a; Parent *et al.* 2011). Protection of airway, acute control of blood loss and maintenance of systemic blood pressure and oxygenation are basic managements for patients with acute SCI to limit the extent of injury and to prevent further complications (Stahel *et al.* 2012). Recent studies also have suggested early and prompt transfer of SCI patients to specialized SCI care centers to decrease mortality rates and medical complications (Divanoglou *et al.* 2010a; Divanoglou *et al.* 2010b; Fehlings *et al.* 2011).

Surgical strategies

Often a surgical decompression with or without arthrodesis is performed to prevent additional neurological damage after an SCI (van Middendorp *et al.* 2012). Clinical trials have indicated a benefit by early decompression therapy, based on an operational definition of "early" being less than 24 hours after SCI (Hawryluk *et al.* 2008). New surgical treatments are under development, such as building bridges with bioengineered scaffold in combination with cell transplantation or neurotrophic factor delivery to provide guidance cues for possible re-establishment of damaged axonal connections (Kubinova and Sykova 2012).

Physical means

Positive results have been reported after hyperbaric oxygen treatment in SCI by partially compensating the reduction of oxygen perfusion (Cristante *et al.* 2012; Kelly *et al.* 1972). Physical exercise including walking, bicycling and step training on a treadmill can result in increased functional recovery, coordination and neurological

performance due to remodeling of intrinsic neural circuit and elevated release of intrinsic growth factors (Ferreira *et al.* 2010; Foret *et al.* 2010; Houle and Cote 2013). Functional electrical stimulation is applied for SCI patients, and has resulted in increased muscle mass, enhanced cardiovascular function, improved bowel function, decreased spasticity and improved body weight-supported walking (Gater *et al.* 2011). Other strategies such as hypothermia are still under evaluation (Hawryluk *et al.* 2008).

Neuroprotective agents

Significant efforts have been devoted to try to limit the evolution of secondary damage after a SCI. High dose methylprednisolone has been relatively widely applied in SCI after reports on its multiple protective effects, including anti-inflammatory action, increase of blood flow, stabilization of cell membranes, inhibition of lipid peroxidation and reduction of glutamate and free radical release (Bracken *et al.* 1992). However, a limited if any functional improvement has been observed in the clinic by methylprednisolone. Therefore, the deleterious side effects from corticosteroids have led many centers to avoid routine usage of corticoids, particularly methylprednisolone, for SCI patients (Domingo *et al.* 2012). Gangliosides induce neuronal regeneration, and increase the *in vitro* formation and growth of neurites as well as the establishment of new functional connections. Improvements of motor and sensory functions have also been reported in SCI patients who received GM1 ganglioside compared to placebos (Hawryluk *et al.* 2008). The combination of GM1 and physical therapy improved motor scores, walking velocity and distance over a placebo or physical therapy alone in individuals with incomplete SCI (Domingo *et al.* 2012; Rabchevsky *et al.* 2011). Another potent neuroprotective drug is the tetracycline antibiotic minocycline, that decreases glutamate-mediated excitotoxicity, acts as an immunomodulator by blocking microglial activation and reduces oligodendrocyte death and axonal dieback in rodent models of SCI (Rabchevsky *et al.* 2011). Other immunosuppressants (e.g. cyclosporine A, rapamycin) were also found to exert neuroprotective effects by inhibiting microglial proliferation and secretion of neurotoxic substances and thereby improving preservation of CNS structures (Hailer 2008). Blocking pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6) in the injured spinal cord to limit secondary damage and to decrease the lesion size is yet another strategy that has resulted in reports of functional improvements (Lavine *et al.* 1998; Mukaino *et al.* 2010; Nesic *et al.* 2001; Okada *et al.* 2004; Vogt *et al.* 2008).

Blocking inhibitory factors

Intact and injured CNS myelin contains several axon growth inhibitory molecules (including Nogo-A, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein). Multiple therapies have been developed to inhibit CSPGs, target and overcome myelin related inhibitors for axon growth. Antibodies against Nogo-A may promote growth of corticospinal tract axons in rats and monkeys after SCI, and even in SCI patients according to a recently performed clinical trial with improved outcome (Pernet and Schwab 2012). The delivery of the enzyme chondroitinase ABC, which inhibits the synthesis of CSPGs, alone or in the combination with physical exercise or cell transplantation, has been reported to increase regeneration of injured axons and to improve functional recovery in experimental models (Bradbury and Carter 2011; Chau *et al.* 2004). In addition, inhibition of Rho-kinase has resulted in protective effects and accelerated functional recovery in experimental acute SCI (McKerracher and Higuchi 2006).

Neurotrophic factors

Neurotrophic factors modulate the survival of neural cells, synaptic plasticity and neurotransmission. Exogenous administration of neurotrophic factors has been proposed as a potential therapeutic approach for SCI. BDNF, GDNF, NT-3, NT-4 and nerve growth factor have all demonstrated therapeutic potential in experimental SCI models (Thuret *et al.* 2006). However, neurotrophic factors alone seem not to be sufficient in assisting injured neurons to overcome the inhibitory environment present in the lesioned spinal cord (Schwab *et al.* 2006). Functional recovery has been achieved by experimental cell transplantation, with olfactory ensheathing cells (OECs), bone marrow mesenchymal/stromal cells (BMSCs), NPCs or ESCs, that were genetically modified to produce neurotrophic factors (Thuret *et al.* 2006).

Cell-based transplantation

Cell-based therapy is another promising strategy for SCI, and pre-clinical researchers have demonstrated that cell-based transplantation can ameliorate secondary events and restore lost tissues. Different sources and types of cells/tissues, including spinal cord and peripheral nerve tissues, stem/progenitor cells (ESCs, induced pluripotent stem cells (iPSCs), NPCs or BMSCs) and non-stem derived cells (Schwann cells,

OECs and activated macrophages), have been and/or are being tested in preclinical stage and clinical trials for SCI (Tetzlaff *et al.* 2011).

Grafts of human fetal spinal cord tissues have been reported to survive up to at least 12 months after transplantation and withdrawal of immunosuppressant, and to obliterate parts of the posttraumatic cyst in patients (Falci *et al.* 1997; Wirth *et al.* 2001). David and Aguayo demonstrated that injured CNS axons were able to regenerate for long distances toward the targeted area with a supportive substrate such as a segment of peripheral nerve (David and Aguayo 1981). Cheng *et al.* transplanted multiple autologous intercostal nerve grafts combined with acidic FGF to bridge the complete spinal cord lesion. A partial restoration of hind limb function in rats was achieved (Cheng *et al.* 1996). Since then, many further attempts have been made by using peripheral nerve grafts combined with neurotrophic factors and/or chondroitinase to repair the injured spinal cord. Axonal regeneration and functional recovery has been shown (Cote *et al.* 2011). Further improvement of the surgical technique and introduction of a biodegradable device to hold the peripheral nerve grafts and to direct the regenerating axons to the grey matter, has resulted in improved functional recovery and motor evoked potentials (Nordblom *et al.* 2012; Nordblom *et al.* 2009).

Schwann cells form the myelin sheaths around the peripheral nerves. They are able to remyelinate the injured axons, promote axonal regeneration by release of growth factors and other growth promoting substances such as laminin and fibronectin (Bozyczko and Horwitz 1986; Cornbrooks *et al.* 1983; Oudega and Xu 2006; Wiliams and Bunge 2012). Grafted Schwann cells are also thought to recruit endogenous host Schwann cells into the injured spinal cord (Biernaskie *et al.* 2007; Hill *et al.* 2006). However, Schwann cells alone seemed not to promote outgrowing axons to overcome the inhibiting glial scar, and hence combination strategies are needed to yield better results (Bunge 2008; Oudega and Xu 2006; Pearse *et al.* 2007). A recent clinical study in eight patients with complete (American Spinal Injury Association Impairment scale A (ASIA-A)) chronic SCI in Iran evaluated the safety and outcome of autologous transplantation of Schwann cells and BMSCs. No adverse observations were reported in any of the enrolled patients up to 2 years following transplantation (Yazdani *et al.* 2013).

In rats, recovery of hind limb function has been reported after transplantation of activated macrophages. The macrophages were activated by incubation with skin tissue or peripheral nerve segments (Bomstein *et al.* 2003; Rapalino *et al.* 1998). Activation of intrinsic macrophages in the injured spinal cord by IL-12 was also reported to benefit a functional recovery in a mice model (Yaguchi *et al.* 2008). It has been suggested that activated macrophages might be a therapeutic target for SCI. In a non-randomized Phase I study performed in Israel, ASIA-A patients with SCI in C5-T11 were given activated autologous macrophages into the spinal parenchyma at the border of the lesion within 14 days after injury. The results showed that the cell therapy was well tolerated in patients (Knoller *et al.* 2005). However, a recent phase II randomized controlled multicenter trial reported that transplantation of activated autologous macrophages into ASIA-A to C patients with acute complete C5-T11 injuries failed to show an improved functional outcome (Lammertse *et al.* 2012).

OECs are glial cells found in the nerve fiber layer in the olfactory bulb, in the nasal olfactory mucosa and surrounding the cranial olfactory nerve fibers, with the property to grow throughout life (Graziadei and Graziadei 1979). OECs are relatively easy to obtain from nasal biopsies and could provide an autologous cell source for grafts. Transplantation of OECs into the injured spinal cord has resulted in increased axonal growth, modulation of the inflammatory response and better functional recovery after SCI (Kubasak *et al.* 2008; Munoz-Quiles *et al.* 2009; Ramon-Cueto 2011). However, the ability of OECs to migrate and to guide re-growing axons appears to be limited by scar tissue (Deng *et al.* 2006; Lee *et al.* 2004). In addition, it is unclear whether OECs can be expanded to sufficient numbers for the purpose of cell therapy. Clinical trials have proved the safety of OECs transplantation in SCI (Feron *et al.* 2005; Lima *et al.* 2006), however, the efficiency of the treatment was not encouraging (Radtke *et al.* 2008).

The most common non-neural cell type applied in experimental or clinical SCI transplantation therapies is BMSCs. The BMSCs are easy to access for autologous transplantation and have relatively well-known immunomodulatory properties. BMSCs have been suggested to provide support for axonal regeneration and improve functional recovery following experimental SCI (Ankeny *et al.* 2004; Chopp *et al.* 2000; Ide *et al.* 2010; Neuhuber *et al.* 2005; Ohta *et al.* 2004; Zurita and Vaquero 2004). Better results were achieved with BMSC injections one week after injury than

immediately after injury (Hofstetter *et al.* 2002). A number of completed and ongoing clinical trials involving transplantation of BMSCs in SCI have reported the feasibility of this treatment, while the functional outcomes vary from different studies (Mothe and Tator 2012). However, in addition to these beneficial effects, there are recently reported adverse effects of BMSCs, such as enhanced tumor growth and metastases (Ramasamy *et al.* 2007; Zhang *et al.* 2013). Thus, further and more careful and detailed studies on clinical safety and efficacy of BMSCs should be performed.

Human pluripotent ESCs have the ability to proliferate for a long period under *in vitro* conditions and have the potential to differentiate into any cell types of the three germ layers, including specific cells of neuronal or glial fates. This enables them to give rise to potential neural cells for application in SCI. Pre-differentiated ESCs transplanted into the injured spinal cord survived, differentiated into neurons and glial cells and showed partial functional recovery (Keirstead *et al.* 2005; McDonald *et al.* 1999; Nistor *et al.* 2005; Sharp *et al.* 2010). However, the application of ESCs has been limited due to the difficulty of generating high-purity lineage-specific cell lines without risk of tumorgenesis and without ethical issues. The generation of iPSCs provides an alternative therapeutic approach for cell therapy in SCI. The use of autologous iPSCs derived from the patient's own somatic cells represents a cell source which is ethically acceptable for most stake-holders, and may mitigate the need for immunesuppression. Transplantation of iPSC derivates into the injured rodent spinal cord showed improvement of locomotor recovery with synapse formation, axon regeneration, increased remyelination and angiogenesis (Fujimoto *et al.* 2012; Nori *et al.* 2011; Tsuji *et al.* 2010). However, iPSC differentiation to neural lineages may occur at a much lower frequency than ESCs (Hu *et al.* 2010). Some types of iPSC-derived neural cells also involve the risk of tumor formation after transplantation. Thus, it is still a big challenge to produce safe iPSC-derived neural cells for transplantation.

NPCs are multipotent cells committed to the neural lineage that can self-renew and be readily expanded *in vitro*. NPCs are of particular interest for SCI repair. NPCs, derived from adult and fetal central nervous system (CNS) tissues, as well as from ESCs and iPSCs as mentioned above, have been reported to survive, differentiate into neurons and glial cells and to improve functional recovery after transplantation in SCI animal models (Amemori *et al.* 2013; Fujimoto *et al.* 2012; Sandner *et al.* 2012;

Tetzlaff *et al.* 2011; van Gorp *et al.* 2013). The mechanism(s) behind the improvement in grafted NPCs is only now beginning to be understood, and several potential mechanisms have been suggested (see details in section 1.4). Adult NPCs have been reported to present limited replication potential (Doetsch *et al.* 1999; Morshead *et al.* 1998) and decreased differentiation potential with time in culture (Wright *et al.* 2006), which would limit clinical application. In addition, the ethical and practical challenges and concerns imposed by the NPC origins and derivation should not be ignored. Nevertheless, a part of the scientific community still believes that NPCs represent an ideal candidate for cell-based treatment of SCI due to functional improvement noticed after transplantation. Finally, NPC populations deriving from the already formed nervous system have not been reported to cause tumor formation with metastasis after transplantation, which is an important criteria for a donor cell population in cell therapy.

Despite all the strategies described above with a promising therapeutic potential in experimental SCI, very few translational studies have resulted in functional improvement in the clinic. Therefore it is important and necessary to continue the search for and development of potent SCI treatments.

1.4 Potential therapeutic effects of transplanted neural cells

Extensive studies have reported the structural and functional restoration from transplantation of neural cells in SCI (Donnelly *et al.* 2012; Nakamura and Okano 2013; Ruff *et al.* 2012; Sandner *et al.* 2012; Tetzlaff *et al.* 2011). Neural cell therapies, contrary to single-molecule-based pharmalogical interventions, hold the potential to deliver a complex series of factors and signals to a multitude of targets in the diseased microenvironment.

Cell replacement

After SCI, a significant loss of neural cells occurs and contributes to the functional deficits (Dumont *et al.* 2001). Thus, replacement of lost or damaged neurons is an important goal in neural cell transplantation. A key prerequisite for neuronal replacement is that grafted stem/progenitor cells differentiate into appropriate neural cells. Transplanted NPCs have been shown to differentiate into neurons (Cummings *et al.* 2006; Cummings *et al.* 2005; Ogawa *et al.* 2002; Yan *et al.* 2007),

oligodendrocytes (Karimi-Abdolrezaee *et al.* 2006; Setoguchi *et al.* 2004) and astrocytes (Cao *et al.* 2001), and result in improved function outcome.

Remyelination

The loss of oligodendrocytes at and around the lesion site, the resulting wide spread demyelination and the loss of appropriate nerve conduction significantly contribute to functional deficits after SCI (Hulsebosch 2002). Regenerative strategies aimed at remyelination represent a promising way to functional restoration after SCI. Pre-differentiated oligodendrocyte progenitor cells (OPCs) or mature oligodendrocytes from ESCs were reported to remyelinate spared axons and improve locomotor function, when transplanted into the injured spinal cord (Keirstead *et al.* 2005; Nistor *et al.* 2005; Sharp *et al.* 2010). Another study also described the restoration of motor-evoked potentials and compound motor action potentials that resulted from the remyelination after NPC transplantation into the contused spinal cord (Yasuda *et al.* 2011). Transplanted human NPCs were also reported to support axon remyelination (Xu and Onifer 2009).

Axonal regeneration

The failure of injured axons in the CNS to regenerate leads to permanent paralysis and other functional deficits after SCI. Numerous studies have examined transplantation of different cell types to create a permissive environment for axon regeneration. Spinal cord-derived NPCs (SC-NPCs) grafted into the transected rat cervical dorsal column elicited enhanced re-growth of corticospinal axons for a limited distance into the graft. Co-localization of regenerating axons with graft-derived GFAP-expressing astrocytes suggested that NPC-derived astrocytes can provide a cellular scaffold for injured axons (Pfeifer *et al.* 2004). Similar mechanisms have been described when glial restricted precursor (GRP)-derived astrocytes were grafted into the injured rat spinal cord, resulting in a superior locomotor recovery determined by grid-walk analysis (Davies *et al.* 2006). The lesion site contains multiple inhibitors of axon outgrowth, such as CSPGs and myelin derived inhibitors as described above (see section 1.2). Hill and colleagues impressively showed that acutely transplanted GRPs both decreased astrocytic scarring and proteoglycan deposition 8 days after contusion resulting in an increased number of corticospinal tract axons (Hill *et al.* 2004).

Synaptic formation

In addition to axonal regeneration, another important regenerative strategy for SCI is to promote synaptic formation and connection. Bonner et al. grafted a mix of GRPs and neuronal progenitors into the injured dorsal column, and observed that the donor progenitors formed a neuronal relay by extending active axons across the SCI to the intended target. Furthermore, synaptic connections between regenerating host axons and graft-derived neurons were established (Bonner *et al.* 2011). Two additional studies also reported that NPC-derived neurons formed new synaptic contacts with host cells after SCI followed by significant functional recovery (Lai *et al.* 2013; Lu *et al.* 2012).

Stimulation of endogenous repair

After SCI, endogenous NPCs, located in the white matter parenchyma or close to the central canal of the spinal cord, are responsive to injury (Obermair *et al.* 2008). Endogenous NPCs were reported to proliferate and differentiate into mature remyelinating oligodendrocytes and/or astrocytes (Ke *et al.* 2006; Obermair *et al.* 2008). However, endogenous NPCs did not lead to complete recovery in cases of severe trauma due to the limited proliferation and differentiation capacity. In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, Einstein *et al.* demonstrated that grafted NPCs stimulated resident OPC proliferation and enhanced remyelination (Einstein *et al.* 2009). Recently, it was also reported that transplanted NPCs increased the neurogenesis from endogenous NPCs in the subgranular zone of dentate gyrus (Jin *et al.* 2011) and subventricular zone in the brain (Mine *et al.* 2013).

Neuroprotection

Since secondary degenerative processes after experimental SCI proceed for at least a few weeks in rats, an important mechanism is a neuroprotective effect by neural cell therapy in the acute and subacute stage to rescue vulnerable neural cells. In later stages, transplanted cells may provide trophic support to promote survival of host cells at the lesion site, and/or supply the injured tissue with an extracellular matrix that is more permissive to regeneration. Lu *et al*. showed that, when grafted to cystic dorsal column lesions in the cervical spinal cord of adult rats, NPCs produced neurotrophic factors and protected host axons (Lu *et al.* 2003). Similarly, it was reported that NPCs protected motor neurons against excitotoxicity in spinal cord

organotypic cultures via release of trophic factors (Llado *et al.* 2004). NPCs may also interfere with the production of free radicals by the release of neuroprotective factors and therefore regulate the oxidative stress occurring after SCI (Madhavan *et al.* 2008).

Immunomodulation

Inflammation characterized by infiltration of peripheral immune cells and activation of microglia play an important role in the pathophysiological development of SCI as described above. In EAE, NPCs were reported to inhibit T-cell proliferation and promote apoptosis of encephalitogenic CNS-infiltrating T cells through apoptotic receptor ligands (e.g. FasL, Apo3L) and release of soluble mediators (e.g. NOS, leukemia inhibitor factor) in the chronic inflammatory environment (Einstein *et al.* 2007; Einstein *et al.* 2006; Pluchino *et al.* 2005). Reduction of the microglial/ macrophage response was observed in stroke after NPC allotransplantation in rodent models, with improved locomotor functions (Bacigaluppi *et al.* 2009; Lee *et al.* 2008). Furthermore, subacute transplantation of NPCs in a stroke model led to significant down-regulation of inflammation-related genes, including IFN-γ, TNF-α, IL-1β, IL-6 and leptin receptor (Bacigaluppi *et al.* 2009). However, relatively few studies have been conducted that concern the anti-inflammatory and immunomodulatory effects of NPCs in SCI and in particular human donor NPCs. Grafted mouse NPCs in SCI mice were reported to migrate to the injury site and synergistically promote functional recovery via modulation of the nature and intensity of the local T cell and microglial response (Ziv *et al.* 2006). Cusimano and co-workers found that subacutely transplanted mouse NPCs reduced the proportion of "classically-activated" M1 macrophages and increased that of regulatory T cells (Tregs), in turn promoting repair of the injured spinal cord (Cusimano *et al.* 2012). Grafted human ESC-OPCs in the EAE mice were able to induce the infiltration of $TREM2^{+}$ CD45 cells, which are specific for tissue regeneration and clearing of cell debris (Kim *et al.* 2012a). Transplanted human GRPs were also shown to ameliorate EAE paralysis by inhibiting T cell proliferation and the activation of macrophages and/or microglia in the brain (Kim *et al.* 2012b).

1.5 Immunogenicity of NPCs and their interactions with immune cells

The immunogenicity of a donor cell population is describing its ability to elicit a host immune response after transplantation. Transplantations can be categorized depending

on donor and host cell or tissue origin. A xenogeneic transplantation is the transplantation of living cells, tissues or organs to a recipient from another species than the donor. Allogeneic transplantation is the transfer of cells, tissues or organs to a genetically non-identical recipient from the same species as the donor. A syngeneic transplantation is cell or tissue transfer between genetically identical individuals, while in an autologous transplantation, donor and host is the same individual.

A rejection response to allogeneic cells is the result of interactions involving coordination between both the innate and adaptive immune system with T cells playing a central role. A rejection is initiated by recognition of antigens on donor cells by T cells via the direct pathway to recognize intact non-self MHC molecules present on the cell surface, or by the indirect pathway including the presentation of processed donor MHC molecules presented as peptides by self-MHC molecules (Ingulli 2010). Xenograft responses differ from alloresponse by comprising hyperacute and delayed rejection. Hyperacute rejection is mediated by xenoreactive natural antibodies and activation of the recipient's complement system. Delayed xenograft rejection is caused by natural antibodies and cellular xenograft rejection involving either T cells or cells from the innate immune system, or both (Yang and Sykes 2007). The rejection of cellular xenografts is stronger than an allorejection (Buhler and Cooper 2005).

The presence of human leukocyte antigen (HLA) class I and II molecules (Johansson *et al.* 2008; Laguna Goya *et al.* 2011; Odeberg *et al.* 2005; Ubiali *et al.* 2007) in conjunction with co-stimulatory molecules CD40, CD80 and CD86 (Odeberg *et al.* 2005) on human NPCs implies a risk for recognition and rejection by non-compatible alloreactive immune cells after transplantation. Varying results have been reported in studies analyzing allogeneic T-cell proliferation stimulated by human NPCs and their derivates from different origins in *in vitro* assay. Previous study showed that human NPCs derived from first trimester nervous system (hfNPCs) did not trigger an allogeneic lymphocyte response (Odeberg *et al.* 2005). Similar findings on human ESC-derived oligodendrocytes were reported by Okamura *et al*. (Okamura *et al.* 2007). In contrast, human NPCs derived from ESCs (hESC-NPCs) or fetal forebrain elicited significant T cell proliferation (Preynat-Seauve *et al.* 2009; Ubiali *et al.* 2007). The possibility to derive human neural cells from iPSCs (Chambers *et al.* 2009; Karumbayaram *et al.* 2009; Takahashi and Yamanaka 2006) or by direct induction of non-neural differentiated cells to neural cells (Vierbuchen *et al.* 2010) offers autologous

cell therapy with reduced ethical concerns and lessened risk of rejection. However, even grafted iPSCs can induce a T-cell-dependent immune response in syngeneic recipients (Zhao *et al.* 2011). Irrespective of their origin, *in vitro* modified and expanded donor cells may present changes in cellular characteristics and protein pattern, putting them at risk of being rejected.

The interaction between neural and immune cells is complex. Xenografted human NPCs from different origins were partly or fully rejected with or without immunosuppression (Hovakimyan *et al.* 2012; Jablonska *et al.* 2013; Wennersten *et al.* 2006). Allogeneic NPCs transplanted into the injured rat spinal cord were subjected to chronic host rejection, though long-term survival of grafted NPCs was still observed (Xu *et al.* 2010). In contrast to triggering immune responses, grafted NPCs may also interact with immune cells to counteract a deleterious inflammatory environment in rodent neurological disease models (Bacigaluppi *et al.* 2009; Cusimano *et al.* 2012; Daadi *et al.* 2010; Einstein *et al.* 2007; Lee *et al.* 2008). However, it is not fully understood how these still immature neural cells exert their immunomodulatory effects and whether similar immunomodulation can take place in an allogeneic human situation.

Grafted mouse NPCs in an EAE model protected against chronic host neural tissue loss as well as disease-related disability by inducing apoptosis of blood-born CNS-infiltrating encephalitogenic T cells (Pluchino *et al*. 2005). It was further shown that NPCs selectively increased the apoptosis of pro-inflammatory T helper 1 (Th1) and Th17 (but not anti-inflammatory Th2) cells *in vitro* through the apoptotic receptors FasL, TRAIL and Apo3L (Knight *et al*. 2011; Pluchino *et al*. 2005). Rodent NPCs inhibited allogeneic T cell activation and proliferation in response to T cell receptor-mediated stimuli (Einstein *et al*. 2003; Fainstein *et al*. 2008), and the inhibitory effect of NPCs may partly depend on the inhibition of IL-2 and IL-6 signaling on T cells (Fainstein *et al*. 2008), the production of heme oxygenase-1 (Bonnamain *et al*. 2012), nitric oxide and prostaglandin E2 (Wang *et al*. 2009). A recent study described that mouse NPCs were able to selectively inhibit pathogenic Th17 cell differentiation by the production of leukemia inhibitor factor, which antagonized IL-6-induced Th17 cell differentiation through the extracellular signal-regulated MAP kinase-dependent inhibition of signal transducer and activator of transcription-3 phosphorylation (Cao *et al*. 2011). Human NPCs suppressed the proliferation of non-human primate or

rodent activated T cells *in vitro* or in EAE model through both direct cell-to-cell contacts and via the release of soluble mediators such as TGF-β (Kim *et al*. 2009; Pluchino *et al*. 2009a; Ubiali *et al*. 2007). In addition, NPCs were reported to restrain the maturation of myeloid dendrite cells via a bone morphogenetic protein-4-dependent mechanism in both *ex vivo* and *in vitro* experiments (Pluchino *et al*. 2009b). Allogeneic hESC-NPCs in an *in vitro* study also induced a strong natural killer cells cytotoxic response, which was driven by activating NKG2D receptor (Preynat-Seauve *et al*. 2009).

Cusimano *et al.* showed that transplanted mouse NPCs interacted with host macrophages via connexin43^{+} cellular junction and reduced the proportion of M1 macrophages, leading to a reprogramming of the local inflammatory cell microenvironment from a "hostile" to an "instructive" state, thus promoting the repair of the injured spinal cord (Cusimano *et al.* 2012). Mosher *et al*. reported that both mouse and rat NPCs regulated microglial functions and activity at least partly through the production of vascular endothelial growth factor by NPCs (Mosher *et al.* 2012). Recently, Cusulin *et al.* showed that mouse ESC-NPCs could fuse with rodent microglia with retained genetic and functional characteristics, at least partly through interaction between phosphatidylserine exposed on the NPCs and the CD36 receptor on microglia (Cusulin *et al.* 2012). However, the interplay of human NPCs and microglial cells still to a large degree is unclear.

As mentioned above, donor NPCs are able to interact with immune cells, even exert immunomodulation on the grafted hosts. However, it needs to be noticed that, most current studies graft human NPCs into genetically immunodeficient animals to investigate the cellular basis of functional recovery without having a rejection response. Thereby, the question, how the intact immune system will interfere with human NPCs short and long term, remains unanswered. Discovery of biological and clinical signs of ongoing rejection in patients with Huntington's disease following allogeneic fetal neural grafts (Krystkowiak *et al.* 2007) underlined the importance of considering immune responses in the CNS as a crucial parameter for cell transplantation strategies. In other studies, immunocompetent rodents are employed followed by immunosuppression to prevent cellular rejection after transplantation of human donor cells, which leads to other issues, such as the differences between xenograft response and allorecognition as mentioned above. Clinical trials including

neural transplantation in Parkinson's disease and SCI patients have also reported survival of allografted human donor tissues despite withdrawal of immunosuppressive treatments (Falci *et al.* 1997; Piccini *et al.* 2005). However, in the allograft trial in Huntington's disease patients mentioned above, a delayed rejection response was observed (Krystkowiak *et al.* 2007). The failure of clinical trials may in part be due to the underestimation of the complexity of the interaction of the human nervous and immune system, as well as the type and duration of immunosuppressant used. Therefore, preclinical studies, with experimental models that are more close to the clinical situations, are needed to bring up more reliable and strong preclinical evidence.

For successful clinical neural cell transplantation, we need to expand our knowledge on how immune cells influence the function of donor NPCs and their progeny in the host tissue. Allogeneic transplantation of ESC-NPCs into intact mouse brain causes the accumulation of host microglia/macrophages and lymphocytes around the graft, which suppressed neuronal differentiation of grafted NPCs by producing cytokines such as IL-6 (Ideguchi *et al*. 2008). In coherence, Gomi and colleagues showed similar phenomenon after allogeneic ESC-NPCs implantation, and further demonstrated that blockade of the accumulation of $CD8^+$ T cells, as well as reduction of the levels of IL-6, resulted in an increased percentage of neurons (Gomi *et al*. 2011). Finally, *in vitro* studies have also indicated that the microglia activation states could differently affect NPCs (Butovsky *et al*. 2006; Cacci *et al*. 2008; Gu *et al*. 2011).

1.6 Human neural cell therapy in SCI clinical trials

Multiple clinical trials including allogeneic human neural cell or solid tissue transplantation therapy have been performed up to date (see section 1.2). Here three important studies including *in vitro* expanded human donor neural cells will be described.

Based on promising preclinical data of human ESC-OPCs in rodent SCI models (Keirstead *et al.* 2005; Sharp *et al.* 2010), the US Food and Drug Administration approved the first human ESCs trial in 2009. A phase I multicenter trial was performed by Geron Corp. (clinicaltrials.gov identifier: NCT01217008) starting in 2010. Patients

with complete thoracic SCI (ASIA-A) were injected with 2 million human ESC-OPCs (GRNOPC1) into the spinal cord lesion site subacutely (between 7-14 days after injury). The enrolled patients will be followed for 15 years after transplantation. However, Geron has discontinued the trial after five patients having received GRNOPC1 therapy and 45-60 days of immunosuppression with tacrolimus (Prograf). No safety issues were reported in any of the grafted patients, but complete results have not been published.

Preclinical studies of human fetal brain-derived NPCs, that differentiated into myelinating oligodendrocytes and synapse-forming neurons as potential mediators of functional recovery in experimental SCI models (Cummings *et al.* 2005; Hooshmand *et al.* 2009; Salazar *et al.* 2010), provided the foundation for a phase I/II clinical trial started by StemCells Inc. (clinicaltrials.gov identifier: NCT01321333) in Switzerland from 2011. The trial is designed to assess the safety and preliminary efficacy in 12 patients with thoracic SCI with variable degrees of paralysis (ASIA A-C), 3–12 months post-injury. Patients receive human CNS-derived NPCs (HuCNS-SC) directly into the injury site and are given 9 months of immunosuppressive drugs, following a 12-month period to monitor safety and potential changes in sensation, motor and bowel/bladder function. Up till now, three ASIA A patients have been enrolled with no complications or side effects reported. Signs of segmental improvement have been observed in two out of the three patients (Guzman *et al.* 2013).

Another phase I multicenter trial using human spinal cord-derived neural stem cells (HSSC) has been proposed and sponsored by Neuralstem Inc. (clinicaltrials.gov identifier: NCT01772810) in USA since Jan. 2013. The trial is designed to evaluate the safety and the graft survival in eight patients with complete thoracic SCI (ASIA-A) at T2-12. Patients will be divided into two groups and receive different amount of HSSC at 1-2 years post-injury, and 3 months of immunosuppression with tacrolimus (Prograf) and mycophenolate mofetil (CellCept). A total of 60 months of observation will be conducted after transplantation. No patient has been recruited to date (http://www.neuralstem.com/cell-therapy-for-sci).

2 AIMS OF THE THESIS

The overall aim was to understand the host response and interactions between host and donor cells in human neural cell therapy after SCI.

The specific aims of this thesis work were:

- To characterize the immunocompetence and immunogenicity of human NPCs and their interaction with allogeneic peripheral blood mononuclear cells
- To determine the nature of the interaction between human NPCs and allogeneic microglia
- To understand allogeneic and xenogeneic host responses to human neural cell therapy in *in vitro* and *in vivo* models of SCI

3 MATERIALS AND METHODS

3.1 Cell and tissue cultures (Paper I-IV)

3.1.1 Human ESC culture (Paper I)

Human ESCs were derived from the inner cell mass of 5/6-day-old pre-implantation blastocyst-stage embryos obtained after *in vitro* fertilization. The embryos used in this thesis had been disqualified from infertility treatment and was donated for human ESC derivation after informed consent from the couple. The Regional Ethics Committee, Stockholm, Sweden has given approval for the derivation, expansion, and differentiation of hESC lines. Human ESCs were initially derived and cultured using postnatal human foreskin fibroblasts (CRL-2429, ATCC) as feeder cells and serum replacement medium as previously described (Inzunza *et al.* 2005), then the colonies were passaged by mechanical splitting at 5- to 7-day intervals and replated on γ-irradiated (35Gy) fibroblasts. The cell culture medium was changed once per day.

Later, suspension cultures were set up for human ESCs to reduce the risk of feeder cell contamination, by applying the protocol developed by (Steiner *et al.* 2010). Briefly, human ESC colonies were mechanically split and transferred to Neurobasal medium containing 14% knockout serum replacement, 2mM GlutaMax, 50U/ml penicillin/ streptomycin, and 1% non-essential amino acids (all from Life Technologies), supplemented with 20ng/ml human basic FGF (R&D), 25ng/ml activin A, BDNF, NT-3 and NT-4, 10ng/ml each (all from Peprotech), 1μg/ml fibronectin (BD Biosciences), 0.5μg/ml laminin, 0.001% gelatin (both from Sigma) and 1×Nutridoma-CS (Roche). After 6–7 days, the colonies formed spheres and the spheres were thereafter mechanically split every 7–10 days. The medium was changed every other day. Human ESC spheres between passage 4 and 10 were used in this thesis.

3.1.2 Neural induction of human ESCs (Paper I)

The neural induction was performed with human ESC spheres according to the protocol by Chambers *et al.* (2009). Briefly, the initial differentiation medium consisted of human ESC-medium with 10μM TGF-β inhibitor SB431542 (Tocris) and 500ng/ml Noggin (R&D). Upon day 5 of differentiation, increasing amount of N2 medium (25%, 50% and 75%) was added to the human ESC-medium every two days while maintaining 10μM SB431542 and 500ng/ml Noggin. After 8 days, the spheres were cultured in NPC medium (DMEM/F12, 0.6% glucose, 5mM Hepes, 2μg/ml Heparin and 1% N2 supplement, all from Life Technologies) supplemented with 20ng/ml

human epidermal growth factor and 20ng/ml human basic FGF (both from R&D) and mechanically passaged once per week up to 20 weeks after neural induction. All cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. The medium was changed every other day. The hESC-NPCs between passage 12-16 were used in this thesis.

3.1.3 hfNPC culture (Paper I-IV)

Cultures of hfNPCs derived from two regions of human first trimester CNS (5-7.5 weeks of gestation) were used in this thesis: SC-NPCs from the spinal cord, and FBr-NPCs from the sub-cortical forebrain. The procedure was approved by the Regional Ethics Committee, Stockholm, Sweden. Briefly, tissue was retrieved from clinical first trimester routine abortions after informed consent by women undergoing termination of pregnancy. Identified tissue was homogenized and cultured at 100,000-200,000 cells/ml in NPC medium supplemented with 20ng/ml human epidermal growth factor, 20ng/ml human basic FGF and 10ng/ml ciliary neurotrophic factor (all from R&D) as described previously (Akesson *et al.* 2009). The free-floating neurospheres were passaged every 7–14 days by enzymatic dissociation using TrypLE Express (Life Technologies) with fresh medium added twice a week. All cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. hfNPCs between passage 0-10 were used in this thesis.

3.1.4 IFN-γ and/or TNF-α stimulation of human NPCs (Paper I)

Human NPCs were stimulated *in vitro* with 200U/ml IFN-γ (R&D), 10ng/ml TNF-α (R&D) or a mixture of both for three days, with the removal of ciliary neurotrophic factor from the culture medium of hfNPCs. Thereafter, neurospheres were dissociated into single-cell suspension for flow cytometric analysis or for co-culture with peripheral blood mononuclear cells (PBMCs), and the supernatant was collected for cytokine analysis.

3.1.5 Human microglial culture (Paper II)

The human microglia line CHME3 was obtained as a kind gift from Professor M. Tardieu (Neurologie pédiatrique, Hôpital Bicêtre, Assistance publique, Hôpitaux de Paris, Paris, France), and cultured as described previously (Hjorth *et al.* 2010). Briefly, CHME3 microglial cells (hereafter called microglia) were cultured in DMEM/high glucose without sodium pyruvate supplemented with 2mM GlutaMaxII and 10% heat-

inactivated fetal bovine serum (all from Life Technologies) at 37°C and 5% CO_2. Microglia were subcultured at confluence using enzyme-free cell dissociation buffer (Life Technologies).

3.1.6 Human fibroblast culture (Paper II)

Human foreskin fibroblasts (hereafter called fibroblasts) were cultured in Iscovs DMEM medium supplemented with 10% fetal bovine serum (all from Life Technologies) to form a confluent monolayer 37°C and 5% CO_2. The cells were enzymatically passaged once per week using TrypLE Express (Life Technologies).

3.1.7 Co-culture of hfNPCs and microglia (Paper II)

The hfNPCs were differentiated at a concentration of 10,000 cells/cm^2 in poly-D-lysine (0.1mg/ml) and fibronectin (0.1mg/ml, both from Sigma) coated plates or flasks and NPC medium supplemented with 1% fetal bovine serum (without mitogens present) for 4 days, to achieve a NPC population just entering differentiation. To mimic an encounter of naïve host microglial cells with donor hfNPCs, unstimulated microglia were added directly to the cultures of differentiating hfNPCs on day 4, allowing cell-cell contact at a concentration of 10,000 cells/cm^2 and the medium volume of 0.1ml/cm^2. Co-cultures were kept in DMEM/F12 and 1% fetal bovine serum for 24 h to allow microglia to attach. Thereafter, hfNPCs and microglia were co-cultured in serum-free medium for up to 48h to avoid the interference of serum to the subsequent cytokine analysis. hfNPC and microglial mono-cultures were processed as controls. hfNPCs co-cultured, under equivalent conditions in sequence as above, with equal amount of fibroblasts or hfNPCs were also set up as controls for some experiments. On day 7, the cells were harvested for further analysis.

3.1.8 Mixed lymphocyte cultures (MLC) (Paper I, III)

In **Paper I**, human adult PBMCs were prepared by centrifuging peripheral blood on a Ficoll gradient (Lymphoprep, Nycomed Pharma) at 500g without brakes for 20 minutes at room temperature. Human CD4 and CD8 T cells were sorted and purified using anti-human CD3, CD4 and CD8 antibodies by FACSAria™ III (Becton and Dickinson). In **Paper III**, rats PBMCs were isolated at 6-week end point from whole blood using NycoPrep (Axis-shield) according to manufacturer's instructions. Spleenocytes were isolated by homogenizing tissue, washed in phosphate-buffered saline, counted and resuspended.

One hundred thousand PBMCs (responder PBMCs) or T cells from one donor were co-cultured with 100,000 irradiated (20Gy) stimulator PBMCs or T cells from one or five donors. Triplicate samples were cultured in 0.2ml RPMI medium (RPMI 1640 medium supplemented with 100U/ml penicillin, 100mg/ml streptomycin, 2mM L-glutamine and 5% pooled normal human male AB serum, all from Life Technologies) per well in 96-well plates. The cell death, differentiation and expression of HLA and co-stimulatory molecules by human NPCs after 6 days in RPMI medium were analyzed by flow cytometry.

To study the effect of human NPCs on PBMC or T cell proliferation, in **Paper I**, 10,000 or 100,000 irradiated hESC-NPCs or hfNPCs (human NPCs: responder PBMC/T cells = 1:10 or 1:1 ratio) were co-cultured for 6 days at 37°C and 5% CO_2. Neutralizing monoclonal antibody to TGF-β1 (clone 9016, 10ng/ml, R&D) was added to study the effect of TGF-β1 in MLC. In **Paper III**, 50,000 rat PBMCs from moderate SCI groups were co-cultured with 500,000 irradiated (20Gy) hfNPCs. On day 5, 1 mCi/ml of [^{3}H]thymidine was added and the cells were harvested after 24 hours. [^{3}H]thymidine DNA incorporation was evaluated using a semiautomatic harvesting machine (Harvester 96, Tomec) and presented as counts per minute (**Paper I**) or stimulation index (**Paper III**). [^{3}H]thymidine incorporation in PBMCs or T cells co-cultured with equal amount of irradiated autologous PBMCs or T cells served as negative controls, and the thymidine incorporation in PBMCs co-cultured with equal amount of irradiated allogeneic pool PBMCs from five donors served as positive controls. Values below three times the background are considered as negative.

3.1.9 Human organotypic slice cultures (Paper III, IV)

Fresh human spinal cord tissues were sliced at 200μm of thickness. Slices were cultured on a semi-porous membrane (pore size 0.4μm, Millipore-Merck) pre-treated with poly-D-Lysine (Life Technologies) and in 1.2ml culture medium (DMEM/F12 with Glutamax, 25% inactivated horse serum, 36mM glucose, 15mM Hepes and 25U/ml penicillin/ streptomycin, all from Life Technologies). Tissues were maintained at 37°C, 5%CO_2, 95% humidity, and the medium was changed twice a week.

At 7 days in culture, contusion injury was achieved on slices using the Infinite Horizon impactor (25kdyn force). One 250–350μm spinal cord-derived neurosphere or a sham injection was applied to the surface of the organotypic slice one hour after injury. The

experimental groups were: 1) Sham; 2) Sham+SC-NPCs; 3) SCI; 4) SCI+SC-NPCs. Slice cultures at 7 and 14 days post-injury with or without SC-NPCs were dissociated into single-cell suspensions for flow cytometric analysis.

3.2 Experimental animals (Paper III, IV)

Female Harland Sprague Dawley rats (B-land, Denmark, **Paper III**) and adult immunodeficient athymic female rats (Hsd: RH-rnu/rnu, Harlan, **Paper IV**) were used in this thesis. The experimental animals were housed in an isolated environment of enriched cages with a 12-hour ligh-dark cycle and access to autoclaved water and food pellets *ad libitum*. All experimental procedures were carried out in accordancec with the guidelines provided by The Swedish National Board and NIH for Laboratory animals, and the experimental procedures were approved by the Regional Ethics Committee on Animal Research, Stockholm, Sweden.

3.3 Surgical and transplantation procedures (Paper III, IV)

The experimental rats (170–200g) were injected intraperitoneally with 0.05mg/kg Atropin (NM Pharma AB) 30 minutes before surgery, and anesthetized using Hypnorm (0.22mg/kg fentanyl citrate and 6.8mg/kg fluanisome, Janssen Pharmaceutical) and 3.4mg/kg midazolam (Hoffman-La Roche). Body temperature was kept at 38°C throughout surgery. Lumbar spinal cords were surgically exposed by partial laminectomy of vertebra T13 with a few drops of 20mg/ml lidocain hydrocloride (AstraZeneca) on the exposed spinal cord surface. Spinal cord contusion injury was achieved with an IH spinal cord impactor (Precision Systems and Instrumentation, LLC), the force set to 150kdyn, 200kdyn (**Paper III**) and 175kdyn (**Paper IV**) with no dwell time. For lateral compression injuries (**Paper IV**), a modification of the method of Rivlin and Tator (1978) was used. A bulldog clamp was applied to compress the spinal cord for 30 seconds at the lower half of spinal cord segment L3.

After surgery, the rats were given intramuscular injections of 7μg/kg buprenorphin (Reckitt & Colman) twice a day for 4 days to avoid allodynia, and 15mg/kg trimetoprim sulfa subcutaneously (Intervet International B.V.) to prevent from urinary infection. The urinary bladders were emptied manually twice daily until spontaneous evacuation was present.

Before transplantation, human fetal-derived neurospheres with a diameter between 150 and 300μm were identified, and 10–12 neurospheres were chosen to give a total number of 100,000 cells ± 10% to be transplanted to each rat. The neurospheres were kept in NPC medium without growth factors pending transplantation.

In **Paper III**, hfNPCs (as whole neurospheres) were grafted into the epicenter of the lesion site within 15 min (acute transplantation) or 9 days (delayed transplantation) after SCI. A subgroup of rats were injected intraperitoneally with 10mg/kg cyclosporine A either for the full study period of 6 weeks or the initial 3 weeks, while the rest was injected with equivalent volume of saline 6 days per week. In **Paper IV**, transplantation to dorsal spinal cord contusion injuries was performed 9 days after lesion. For lateral compression injuries, hfNPCs were transplanted 10 minutes (acutely), one week (subacutely) or 7 weeks (chronically) after compression injury.

3.4 Functional assessments (Paper III, IV)

Locomotor behavior was evaluated using the Basso, Beattie, Bresnahan locomotor rating scale (Basso 2004; Basso *et al.* 1995). In **Paper III**, the test was carried out prior to surgery, 7 and 42 days post-surgery (the end point). In **Paper IV**, the rats were evaluated pre-operatively and then on regular intervals for 18 weeks.

In **Paper IV**, hindlimb motor function was also assessed using a grid-walk test. To assess increased pain sensitivity, pain threshold to pressure to the torso and hindlimbs was tested before the injury, immediately after injury and 1, 2, 6, 10 and 18 weeks later, using von Frey filaments (Stoelting) ranging from 0.4 to 447 g (Yu *et al.* 1999).

3.5 Analysis of cell viability and death (Paper I, II, IV)

In **Paper II**, cell viability was assessed by resazurin assay, in which the resazurin sodium salt (20μg/ml, Sigma) was used as indicator of cell metabolic activity. Results are presented as differences in fluorescence intensity of formazan salt. In **Paper I, II and IV**, cell death was evaluated utilizing the necrosis marker 7-AAD and/or intracellular apoptosis marker active caspase-3 by flow cytometry.

3.6 Proliferation assay (Paper II)

Carboxyfluorescein diacetate succinimidyl ester (CFSE)-based proliferation assay, depending on a reduction of CFSE fluorescence intensity in successive generations of a

proliferating cell population, was performed to evaluate the degree of cell proliferation. Briefly, before starting co-cultures, hfNPCs or microglia were labeled with CFSE (5μM, Life Technologies) followed the manufacturer's instruction. hfNPCs and microglia were co-cultured for 3 days, then the percentage of divided cells was analyzed by flow cytometry.

3.7 Phagocytosis assay (Paper II)

To analyze the microglial phagocytic activity, microglial cells were labeled by fluorescent probe 5-(and 6)-(((4-chloromethyl)-benzoyl) amino)-tetramethyl-rhodamine (10μM, Life Technologies). On day 7, cells were incubated with latex beads in fluorescent yellow-green (diameter 0.03μm, 1:1000, Sigma) at 37°C for 2 h. Microglial mono-cultures were set up as control. Afterwards, cells were harvested and microglial uptake of latex beads was analyzed by flow cytometry.

3.8 Immunomodulatory assay (Paper I)

To investigate how human NPCs affect PBMC sub-populations and cytokine secretion in co-cultures, PBMCs, were prepared as described in **3.1.8** and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 1μM, Life Technologies) according to manufacturer's instructions, were co-cultured with equal amount of non-irradiated hESC-NPCs or hfNPCs (5×10^5 cells/well in 6-well plates) for 3 and 6 days. Equivalent cultures were set up in Transwell system with human NPCs and PBMCs separated by a semi-permeable membrane (pore size 0.4μm, Millipore-Merck). The proportion of $CD4^+$, $CD8^+$, $CD4^+CD25^+$ and $CD4^+CD25^+$forkhead box P3 $(FOXP3)^+$ T cells (a subpopulation previously reported by others to identify induced Tregs (Dummer *et al.* 2012)) and dead T cells in co-cultures recognized by 7-amino-actinomycin D (7-AAD) was analyzed by flow cytometry. The culture supernatant was collected for cytokine analysis at day 6.

To study effects of TGF-β1 and TGF-β2 on PBMC proliferation, 150,000 PBMCs were cultured in the presence of phytohaemagglutinin (10μg/ml, Sigma) for 4 days. Human recombinant TGF-β1 and TGF-β2 protein (R&D) were added to the culture at 0.01, 0.1, 1ng/ml and in the case of TGF-β1 also 10ng/ml. The results were analyzed by $[^3H]$thymidine incorporation in PBMCs and presented as percentage of PBMC proliferation in the presence of phytohaemagglutinin without hTGF-β1 or hTGF-β2 protein (set as 100%).

3.9 Flow cytometry (Paper I-IV)

To label surface markers, cells were stained with respective antibodies at 4°C for 30 minutes, washed, resuspended in phosphate-buffed saline and fixed in Cytofix fixation buffer (BD Biosciences). FITC-, PE-, PE-Cy5-, PE-Cy7-, PerCP-Cy5.5-, APC- and Alexa Fluor 647-conjugated isotype antibodies were used as controls. For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm Fixation/ Permeabilization Kit (BD Biosciences) following the manufacturer's instructions. Thereafter, the cells were incubated with respective antibodies at 4°C for 30 minutes, washed and resuspended in phosphate-buffered saline. To analyze intracellular cytokines, Brefeldin A (1:500, BD Biosciences) was added to the culture medium during the last 6 hours to block cytokine secretion, followed by intracellular staining. All antibodies used in flow cytometry in this thesis are listed in Table 1. Flow cytometry data were collected using a FACScalibur (Becton and Dickinson) and data analysis on percentage of positive cells and mean fluorescence intensity was performed with the software FlowJo (Tree Star, Inc.).

Table 1. Antibodies used in flow cytometry (to be continued)

Conjugated antibodies				
Antibodies	**Fluorescence conjugated**	**Clone**	**Manufacturer**	**Paper**
CD3	FITC	UCHT1	Dako	I
CD4	APC	RPA-T4	BD Biosciences	I
	FITC	RPA-T4	BD Biosciences	III
CD8	PE	RPA-T8	BD Biosciences	I, III
	PE-Cy7	RPA-T8	BD Biosciences	I
CD11b	PE-Cy7	M1/70	BD Biosciences	III
CD15	PC5	80H5	Beckman Coulter	I
CD24	PE	ML5	BD Biosciences	I
CD25	PE	M-A251	BD Biosciences	I
CD29	PE-Cy5	MAR4	BD Biosciences	I
CD31	FITC	WM59	BD Biosciences	I
CD40	FITC	5C3	BD Biosciences	I, II, III
	AlexaFluor647	5C3	Biolegend	I
CD44	PE	515	BD Biosciences	I
CD45	APC	HI30	BD Biosciences	III
CD54	PE	HA58	BD Biosciences	I
CD56	PE	MY31	BD Biosciences	I
CD58	FITC	1C3	BD Biosciences	I
CD68	AlexaFluor647	Y1/82A	BD Biosciences	III
CD80	FITC	L307.4	BD Biosciences	I, II, III
	PE-Cy7	L307.4	BD Biosciences	I

Table 1. Antibodies used in flow cytometry (continued)

Antibodies	Fluorescence conjugated	Clone	Manufacturer	Paper
CD86	FITC	2331	BD Biosciences	I, II, III
	AlexaFluor647	IT2.2	Biolegend	I
CD163	AlexaFluor647	RM3/1	Biolegend	II
CD184	PE	12G5	BD Biosciences	I
CD200	PerCP-Cy5.5	MRC OX-104	BD Biosciences	II
CD200R	PE	OX-108	Biolegend	II
CD206	AlexaFluor647	15-2	Biolegend	II
HLA-I	PE	W6/32	Dako	I, III
HLA-II	FITC	CR3/43	Dako	I, II, III
HLA-DR	PE	G46-6	BD Biosciences	I, III
Stage-specific embryonic antigen 4	PE	MC-813-70	Millipore-Merck	I
Polysialylated-neural cell adhesion molecule (PSA-NCAM)	PE	2-2B	Miltenyi Biotec	I, II
A2B5	PE	105-HB29	Miltenyi Biotec	I, II
Nestin	PerCP-Cy5.5	aa. 402-604	BD Biosciences	I, II
GFAP	PE	Cow spinal cord homogenate	BD Biosciences	I, II
β-tubulin III	AlexaFluor647	rat brain microtubules	BD Biosciences	I, II
FOXP3	PerCP-Cy5.5	236a	BD Biosciences	I
TGF-β1	PE	9016	R&D	I, II
7-AAD			BD Biosciences	I, II
Active Caspase-3	PE	C92-605	BD Biosciences	II, IV
Unconjugated primary antibodies				
Antibodies	**Isotype**	**Concentration**	**Manufacturer**	**Paper**
TRA-1-60	Mouse IgM	1:500	Millipore-Merck	I
TRA-1-81	Mouse IgM	1:200	Santa Cruz	I
Brachyury	Rabbit IgG	1:200	Sigma	I
Ki67	Mouse IgG	1:100	Dako	I
TGF-β2	Rabbit IgG	1:200	R&D	II
Unconjugated secondary antibodies				
Antibodies		**Concentration**	**Manufacturer**	**Paper**
Anti-mouse FITC IgM		1:200	BD Biosciences	I
Anti-mouse FITC IgG		1:200	BD Biosciences	I
Anti-rabbit FITC IgG		1:200	BD Biosciences	I, II
Isotype antibodies				
Isotype	**Fluorescence conjugated**		**Manufacturer**	**Paper**
IgM	PE		Miltenyi Biotec	I, II
IgG	FITC/PE/PE-Cy5/PE-Cy7/PerCP-Cy5.5/APC/ AlexaFluor647		BD Biosciences	I, II

3.10 Immunochemistry (Paper I-IV)

In **Paper I**, ten to fifteen intact neurospheres of hESC-NPCs and hfNPCs were collected, fixed in 4% paraformaldehyde for 15 minutes at room temperature, treated with 30% sucrose over night at 4°C, mounted in Tissue-Tek mounting medium (Sakura Finetek), frozen on dry ice and sectioned at a thickness of 5µm. In **Paper II**, cells cultured on cover slips were fixed with 4% paraformaldehyde for 15 minutes at room temperature and rinsed in phosphate-buffered saline. In **Paper III and IV**, the rat spinal cord tissue was fixed for 90 min-2 hours, rinsed and cryopreserved in 10-20% sucrose at 4°C for at least 24 h. The spinal cords were sectioned at 10µm in a cryostat and mounted on gelatin-coated slides.

Cells or tissue sections were pre-incubated with serum at room temperature, incubated with primary antibodies overnight at 4°C, rinsed before incubation with the secondary antibodies at room temperature. All antibodies used in immunochemistry in this thesis are listed in Table 2. The cell nuclei were stained with Hoechst 33342 (1:400, Life technologies) prior to mounting in PVA-DABCO (9.6% polyvinyl alcohol, 24% glycerol, and 2.5% 1,4-diazabicyclo (2.2.2) octane in 67 mM Tris-HCl, pH 8.0). The images were acquired in a fluorescence microscope (Axiophot, Zeiss) and documented using a CCD camera (ORCA-ER) together with the Openlab software 3.1.7 for Macintosh (Improvision), or using an inverted microscope Axiovert 200M (Carl Zeiss MicroImaging GmbH) connected to a LSM510 META confocal unit.

3.11 Quantitative histological analysis (Paper III, IV)

In **Paper III**, to determine the extent of the microglial and macrophage response, the mean intensity of the ED1 immunoreactivity was quantitatively evaluated using Image-Pro Plus software (Media Cybernetics) and calculated as luminosity units per tissue area. In **Paper IV**, the proportion of human donor cells expressing phenotype markers and the number of survived rat spinal cord neurons were evaluated by counting immunoreactive cells in 4–6 fields of view at 40× magnification, and calculated with Abercrombie correction.

3.12 Enzyme-linked immunosorbent assay (ELISA) (Paper I, II)

The ELISA reagents and components for TNF-α, IL-6, IL-10, TGF-β1, TGF-β2, BDNF, GDNF and NT-3 were purchased from R&D. The experimental procedure was performed according to the manufacturer's instructions with Tris-buffered saline as

Table 2. Antibodies used in immunochemical procedures

Primary antibodies				
Antibodies	**Species**	**Concentration**	**Manufacturer**	**Paper**
Nanog	Goat	1:200	R&D	I
Brachyury	Rabbit	1:200	Sigma	I
α-fetoprotein	Goat	1:200	Santa Cruz	I
Pax-6	Rabbit	1:200	Santa Cruz	I
Nestin	Mouse	1:200	Millipore-Merck	I, II
	Rabbit	1:400/1:250	Millipore-Merck	I, IV
β-tubulin III	Mouse	1:800	Covance	I, II
	Rabbit	1:1200	Nordic Biosite	IV
GFAP	Rabbit	1:500	Dako	I
	Mouse	1:50	Abcam	II
	Mouse	1:200,000	Sternberger Monoclonals	IV
Ki67	Mouse	1:100	Dako	I
Iba-1	Rabbit	1:200	Wako	II
ED1	Mouse	1:200	AbD Serotec	III
Synapse-associated protein 102 (SAP102)	Rabbit	1:200	Almone labs	III
Heat shock protein 27	Rabbit	1:1500	Medical & Biological Laboratories	III, IV
Human nuclear protein	Mouse	1:150	Millipore-Merck	IV
Proliferating cell nuclear antigen	Mouse	1:100	Cell Signaling Technology	IV
Microtubule-associated protein 2	Rabbit	1:50	Millipore-Merck	IV
Secondary antibodies				
Anti-goat Alexa Fluor 488	Donkey	1:1200	Life Technologies	I
Anti-mouse Alexa Fluor 488	Goat	1:1200	Life Technologies	I, II, III
Anti-rabbit DyLight 549	Donkey	1:2400	Jackson ImmunoResearch Laboratories	I
Anti-mouse Cy3	Goat	1:2400	Jackson ImmunoResearch Laboratories	I
Anti-rabbit Cy3	Goat	1:2400	Jackson ImmunoResearch Laboratories	I, II, III

wash buffer. In **Paper II**, QuantaRed fluorescent substrate (Thermoscientific) was used instead of a colorimetric substrate in the assays for TNF-α, IL-10, GDNF and NT-3.The culture supernatant were processed in triplicate samples. The analysis of 96-well plates was achieved in a TECAN Safire2 microplate reader (Männedorf) at 540 nm.

3.13 Statistical analysis (Paper I-IV)

Analysis of differences among groups was performed by either parametric (t-test or One-way ANOVA) or non-parametric tests (Mann-Whitney test or Kruskal-Wallis test) depending on whether the data were normally distributed or not. In **Paper III**, Chi-square test for independent groups was used to evaluate differences in rejection ratios between experimental groups. All statistical analysis was performed with Instat 3.0 (GraphPad Software Inc.) or StatView 4.5 (Abacus Concepts) for Macintosh. P-values less than 0.05 were considered statistically significant. Data are shown as mean ± mean standard error (**Paper I-III**), mean ± standard deviation (**Paper IV**) or median ± percentiles (25%-75% and 5%-95%, **Paper II**).

4 RESULTS AND DISCUSSION

4.1 Characterization of human NPCs (Paper I-IV)

We compared cellular characteristics of human NPCs *in vitro* cultured under equivalent conditions but originating from different origins. We observed that hfNPCs and hESC-NPCs expressed similar amounts of a number of markers, such as neuronal progenitor marker PSA-NCAM, CD29 (reported to modulate NPC survival, migration, proliferation and differentiation (Campos 2005)), CD44 (known to be expressed on glial progenitors and astrocytes (Liu et al. 2004)) and neural cell adhesion molecule CD56 (Paper I). In contrast, hfNPCs presented a higher percentage of cells expressing the glial progenitor marker A2B5 and the neural cell growth and migration modulator CD184 than hESC-NPCs. We further showed that, when hfNPCs were cultured with mitogens present approximately 92% were nestin$^+$, while 56% were GFAP$^+$ of the total hfNPCs population. Only 6% of the hfNPCs were β-tubulin III$^+$. After differentiation for 7 days (Paper II), the percentage of hfNPCs expressing nestin decreased, while the subpopulation of cells positive for β-tubulin III and GFAP was significantly increased, These findings indicated that hfNPCs were able to differentiate into both neuronal and glial lineages *in vitro*.

Twenty weeks post transplanttion into SCI rats (Paper IV), hfNPCs positive for the early neuronal marker β-tubulin III and the more mature neuronal marker microtubule-associated protein-2 comprised less than 10% of the total donor cell population. In all groups, the majority (80–90%) of transplanted hfNPCs differentiated into astrocytes (GFAP$^+$ cells), which previously have been associated by others with allodynia (Hofstetter *et al.* 2005; Macias *et al.* 2006). In our case, no allodynia was observed after hfNPC transplantation despite astrocyte differentiation, suggestion it is not astrocyte differentiation in general that is the cause for this severe side effect.

Proteins involved in antigen processing and presentation were studied on hESC-NPCs and hfNPCs. Both hESC-NPC and hfNPC populations presented high percentages of HLA-I$^+$ and CD58$^+$ cells, but expressed low proportions of co-stimulatory molecules CD80 and CD86, as well as adhesion molecule CD54. Cells expressing the co-stimulatory molecule CD40 were more abundant among hfNPCs than hESC-NPCs. However, in neither cell type, no HLA-II$^+$CD40$^+$CD80$^+$CD86$^+$ cells were detected by flow cytometry. The expression pattern of these immune-related markers indicates that

the antigen presentation to induce naïve T cells is limited due to the lack of expression of HLA-II, adhesion and co-stimulatory molecules on the same cells. Moreover, none of these molecules was affected by stimulation of pro-inflammatory cytokine in neither human NPC population, further supporting the hypothesis that hESC-NPCs and hfNPCs have a limited risk of triggering an immune response, even in an ongoing inflammatory milieu, such as a SCI.

It has previously been demonstrated that the duration of *in vitro* propagation of human NPCs may lead to changes in cellular composition even under equal culture conditions (Piao *et al.* 2006). The expression pattern of immune-related proteins on hfNPCs with "short" (P0) and "long" (P5) *in vitro*-expansion time was investigated. P0 hfNPCs expressed low percentage of HLA-I and HLA-II (11% and 4%) compared to P5 cells (85% and 35%). The co-stimulatory molecules CD40, CD80 and CD86 were undetectable at P0, while at P5 expressed in up to 5% of hfNPCs. The observed increased expression of HLA, co-stimulatory and adhesion molecules (Akesson *et al.* 2009; Odeberg *et al.* 2005) on hfNPCs allows them at least in theory, despite an initial limited risk, to act as antigen presenting cells with direct antigen presentation to elicit the host immune reaction.

Cytokines secreted by human NPCs may affect the host immune response in allogeneic neural cell therapy. Compared to hESC-NPCs, unstimulated hfNPCs secreted more TGF-β1 (in Paper I), a factor involved in the maintenance of immune tolerance and T-cell homeostasis (Bommireddy and Doetschman 2007) and also reported to balance the immunogenicity of FBr-NPCs (Ubiali *et al.* 2007). hfNPCs also released higher level of TGF-β2 (in Paper I). Increased TGF-β2 levels may polarize naïve T cells toward a regulatory function that could in turn mediate suppression antagonizing adaptive immune responses (Robertson *et al.* 2007). In addition, the release of TGF-β1, TGF-β2 and IL-10 by hESC-NPCs increased under inflammatory conditions, suggesting that the immunomodulatory potential of hESC-NPCs might be promoted when they encounter an inflammatory environment as in neurodegenerative diseases and CNS trauma.

4.2 Survival of human NPCs after SCI transplantation (Paper III, IV)

Human neural donor cells have been reported to respond differently to host environment cytokines depending on regional origin (Johansson *et al.* 2008). However, no difference was observed in number of survived donor cells between SC-NPCs and

FBr-NPCs transplanted SCI rats (Paper IV). When SC-NPCs *in vitro* expanded for 5 passages were grafted acutely into immunocompetent severe SCI rats, no surviving donor cells were found in the injured spinal cords 6 weeks after transplantation, while SC-NPCs implanted at P0 were still present in 3/5 cases. In the presence of the immunosuppressant cyclosporine A, no difference was observed in the survival of SC-NPCs implanted at P0 and P5. The lower rejection ratio for short-term *in vitro* expanded SC-NPCs may be explained by the fact that fewer NPCs expressed HLA and co-stimulatory molecules after short in vitro culture time (Akesson *et al.* 2009; Odeberg *et al.* 2005).

Levels of potentially toxic cytokines in the injured spinal cord increase rapidly after injury (Nakamura *et al.* 2003; Pineau and Lacroix 2007), which may harm the donor cells. However, this has previously not been systematically investigated. To address this issue, donor human NPCs were grafted at different time point post-injury and the donor survival in the host spinal cord was analyzed. No difference was found on the survival of acutely or delayed grafted SC-NPCs 6 weeks after transplantation in the moderate SCI rats, in the presence of immunosuppressant (Paper III). The survival of human donor cells in the host spinal cord long term (20 weeks) after transplantation was further investigated (Paper IV). Although there was no significant difference among groups with acute, subacute or chronic transplantation, the mean number of human cells was highest in the group transplanted acutely. Hence, the acutely injured spinal cord does not seem to be a more hostile milieu for grafted human NPCs than that in the subacute or chronic phase. The reason may be that NPCs are less sensitive to apoptotic stimuli, as has been found for other neural stem cells (Tamm *et al.* 2004). To further address how the host milieu may influence donor neural cells, host rats were exposed to severe or moderate contusion SCI in Paper III. In the absence of immunosuppression, acutely grafted SC-NPCs was fully rejected in all hosts 6 weeks post-injury in the severely injured hosts, while SC-NPCs implanted with a delay of 7 days into moderate SCI survived in 4/6 rats, suggesting that acute severe injury with a more intense inflammatory response is more detrimental for the donor NPCs. Further studies are needed to provide direct evidence how the SCI severity influences the fate of donor cells, with the same *in vitro* expanded duration and transplantation timing of the donor cells.

Furthermore, the number of donor human cells 20 weeks after transplantation into immunosuppressed SCI hosts greatly exceeded the number of transplanted NPCs, in some animals as much as 9-fold. This contrasts to some earlier transplantation studies (Hofstetter *et al.* 2005; Karimi-Abdolrezaee *et al.* 2006), while other report similar proliferation after transplantation of human neural stem cells to chronic SCI in mice (Salazar *et al.* 2010). We have previously reported that cell death in transplanted neurospheres is surprisingly low, and that some low degree of proliferation of the grafted SC-NPCs continues, but peaks already at 1 day post transplantation and then declines (Emgard *et al.* 2009). This results in a net increase of human cells.

4.3 Host lymphocyte response to human NPCs (Paper I, III)

The presence of HLA, co-stimulatory and adhesion molecules on human NPCs implies a risk for recognition and rejection by non-compatible immune cells after transplantation. In the *in vitro* allogeneic co-cultures of human NPCs and PBMCs, hESC-NPCs triggered PBMC proliferation in a dose-dependent manner, and the response was not affected by IFN-γ and/or TNF-α (Paper I). In contrast, hfNPCs tested under equivalent conditions did not elicit an allogeneic PBMC proliferation, which is consistent with previous findings on hfNPCs and their differentiated derivates (Akesson *et al.* 2009; Odeberg *et al.* 2005). Noteworthy is the report by, Laguna Goya *et al*., that hfNPCs triggered a delayed proliferative response of allogeneic T cells after 14 days *in vitro* (Laguna Goya *et al.* 2011). Despite the fact that hESC-NPCs can trigger PBMC proliferation already after 6 days in co-culture, neither hESC-NPCs nor hfNPCs further enhanced a PBMC alloresponse triggered by a third party of allogeneic PBMCs, but instead hindered the proliferative response. This effect also occurred in a dose-dependent pattern and was altered by the addition of pro-inflammatory cytokines in the cultures. Mesenchymal stem cells are known to have strong immunesuppressive properties on immune cells and can counteract graft versus host disease in clinical hematopoietic stem cell therapies (Le Blanc *et al.* 2008), which requires activation by a pro-inflammatory environment to exert the immunomodulatory effects (Engela *et al.* 2012). Similarly, an activated immunosuppressive process by hESC-NPCs or hfNPCs was found in the ongoing alloresponse milieu (Paper I).

Transplantation of syngeneic mouse NPCs in a focal SCI increased the proportion of Tregs (Cusimano *et al.* 2012), which are known to promote antigen-specific peripheral tolerance by suppressing the activation and expansion of reactive effector cells. In

Paper I, both hESC-NPCs and hfNPCs up-regulated Tregs, defined as the proportion of $CD4^+CD25^+FOXP3^+$ T cells among $CD4^+$ T cells, after 6 days co-culture with direct cell-cell contact but not in the transwell system. This suggests that cell-cell contact between human NPCs and allogeneic PBMCs is an important mechanism to up-regulate $CD4^+CD25^+FOXP3^+$ T cells. The production of immunomodulatory cytokines such as TGF-β1 is one of the major pathways through which $FOXP3^+$ Tregs exert their regulatory function. Bommireddy *et al*. reported that $FOXP3^+$ Tregs in TGF-$\beta1^{-/-}$ mice were not able to inhibit the activation of $CD4^+CD25^+$ T cells, suggesting the signal from FOXP3 alone in Tregs is not enough for their action (Bommireddy *et al.* 2009). It seems as if TGF-β1 is required for the suppressive function of Tregs. In Paper I, the release of TGF-β1 was coherent with the up-regulation of $CD4^+CD25^+FOXP3^+$ T cells in the presence of hfNPCs in direct contact with PBMCs, but not hESC-NPCs. The PBMC proliferative response could be reduced by the exogenous addition of TGF-β protein. Furthermore, the participation of TGF-β1 antibody at least partly interfered and resulted in a higher proliferative response in co-cultures of PBMC and human NPCs. Taken together, these events suggest that TGF-β1 released at high enough levels by human NPCs may support the induced $FOXP3^+$ Treg population to exert their suppressive effects.

The $CD4^+$, $CD8^+$ or $CD25^+$ populations in peripheral blood and spleen of the severe injured xenogeneic hosts in an *in vivo* SCI model were not observed to be affected by grafted hfNPCs. Interestingly, a group of $CD4^+CD8^+$ cells, which have been reported to participate in antigen exposure and lymphocyte maturation (Kenny *et al.* 2004) as well as to play a protective role in clinical acute graft versus host disease (Eljaafari *et al.* 2013), was found in the rodent hosts. When PBMCs from moderate SCI rats and equivalent hfNPCs as grafted *in vivo* were co-cultured in an *in vitro* MLC assay, the stimulation index (related to the PBMC proliferative response) was significantly higher in all lesioned groups with or without donor hfNPCs, compared to the normal control. However, no significant difference could be observed in the lymphocyte populations among the SCI groups despite a known host rejection response in some of the groups.

4.4 Host microglial/macrophage response to human NPCs (Paper II, III)

Microglia are known to be the primary mediators of the innate immune response in the CNS, playing an important role in immune surveillance and homeostasis under normal conditions (Kettenmann *et al.* 2011). Upon injury, microglia are rapidly activated and

become selectively directed to the injured site (Hanisch and Kettenmann 2007). In Paper II, a human allogeneic setting was utilized, by seeding "non-activated" microglia with hfNPCs early during differentiation, to evaluate their effects on each other. The proliferation and phagocytic activity of human microglia were increased in the presence of hfNPCs, as well as the proportion of microglia positive for M1-marker HLA-II or M2-marker CD206. These data suggested that hfNPCs can influence the degree of microglial activation and functional activity *in vitro*, and that different microglial phenotype can emerge under these human co-culture conditions. On the other hand, co-culture with microglia increased the proliferation of hfNPCs, which is in accordance with findings from rodent studies (Cacci *et al.* 2008; Mosher *et al.* 2012), where conditioned medium from untreated microglia were added to NPCs. hfNPCs in co-cultures with human microglia presented a higher percentage of nestin, and a lower proportion of PSA-NCAM, A2B5 and GFAP positive cells compared to mono-cultures. This observation suggested that the presence of microglia inhibited hfNPC maturation and differentiation with regard to both neuronal or astroglial destiny. These observations could be partially explained by the presence of IL-6 released by microglia in the co-cultures as suggested by others (Ideguchi *et al.* 2008). Thus, microglia might play a role as a determinant of NPC destiny in human allogeneic setting.

Glial cells and neurons are reported to express a range of neuroimmune regulatory proteins (NIRegs), such as CD200, to counteract neurotoxic effects of innate immune molecules (Grimsley and Ravichandran 2003; Neumann 2001). NIRegs in analogy to Tregs, are involved in silencing and reshaping an adverse innate immune response, and in polarizing microglia towards a protective phenotype, to limit inflammatory reactions in the injury site (Griffiths *et al.* 2010). CD200R, structurally closely related to CD200, is mainly expressed on myeloid cells such as microglia and delivers an inhibitory signal after ligation to CD200 (Jenmalm *et al.* 2006). The CD200-CD200R interaction provides a negative cell-cell contact dependent regulatory signal for microglia and protects neural cells in neurodegenerative diseases (Chitnis *et al.* 2007; Koning *et al.* 2009; Walker *et al.* 2009; Zhang *et al.* 2011). Therefore, it is reasonable to assume that a CD200-CD200R interaction plays an important role in regulating microglial activity and restricting neuroinflammation. In the allogeneic hfNPC-microglia co-cultures, not only did a larger proportion of hfNPCs express CD200, but also the expression of CD200R on microglia was up-regulated. As a result, the opportunity for a CD200-CD200R interaction between hfNPCs and microglia was increased, which may limit

deleterious neuroinflammation caused by microglia. Blocking the CD200-CD200R interaction can lead to decreased TGF-β expression (Clark *et al.* 2008; Memarian *et al.* 2013), in other words, CD200-CD200R interaction may support the release of TGF-β. In the allogeneic co-cultures, the TGF-β2 level in supernatant was higher than in monocultures, and the subpopulation of TGF-β1 expressing hfNPCs was significantly larger in the presence of microglia. The increased TGF-β release might be due to an enhanced CD200-CD200R interaction between hfNPCs and human microglia. In addition, the increased level of TGF-β may contribute to the enhanced phagocytic activity of microglia in co-cultures, as TGF-β has been shown to enhance survival and activity of phagocytic microglia (Ryu *et al.* 2012; Tichauer and von Bernhardi 2012). The up-regulation of anti-inflammatory M2-marker CD206 might be due to the elevated release of TGF-β2, which may help to polarize microglia towards an anti-inflammatory phenotype and to influence the antigen-presenting functions of microglia (Paglinawan *et al.* 2003; Siglienti *et al.* 2007).

In Paper III, the host microglial and macrophage response to xenotransplanted hfNPCs was evaluated in a contusion SCI animal model through the quantitative analysis of ED1 immunoreactivity. ED1, equivalent to CD68 in humans, is a single chain glycoprotein expressed on lysosomal membranes of myeloid/macrophage cells and highly expressed by phagocytic cells (Damoiseaux *et al.* 1994). In the normal spinal cords, the ED1 immunoreactivity was very low with no observed activated microglia and macrophages present. In the injured spinal cord, numerous amoeboid shaped and intensely immunoreactive $ED1^{+}$ cells were observed. In hfNPC transplanted group, the ED1 immunoreactivity in the moderate injury epicenter was significantly decreased suggesting a reduced microglial/macrophage inflammatory response locally in the SCI epicenter compared to non-grafted rodents. No difference was observed between hfNPC-P0 and hfNPC-P5 grafted groups, or between acute and delayed transplanted groups. Similarly, donor neural cells have been reported to decrease CNS inflammation by immunomodulatory effects in EAE (Einstein *et al.* 2007) and stroke models (Mine *et al.* 2013). With the observed difference in the microglial/macrophage reaction between *in vivo* xenografted and non-grafted groups, the host microglial response was further investigated in a human allogeneic setting by employing an *in vitro* human organotypic spinal cord slice culture model. The immunomodulatory effect by hfNPCs on activated microglia was confirmed in human allotransplantation model mimicking

human allogeneic neural cell therapy. Important to note, allografted hfNPCs *per se,* in the sham control slices, did not trigger sustained microglial activation.

Furthermore, microglia are known to play an active role in the development of mature synapses (Schafer *et al.* 2013). Activated microglia could stimulate either neosynaptogenesis (Bessis *et al.* 2007) or induce synaptic stripping (Kettenmann *et al.* 2013). An attempt was made to evaluate the expression and distribution of the post-synaptic protein SAP102 at the site of microglia ($ED1^{+}$) accumulation in the lesioned epicenter of moderate injured spinal cord, but no sign of changed immunoreactivity in SAP102 could be observed between grafted and non-grafted groups. Further study is needed to investigate the complex interaction between microglia and the neuronal synapse.

4.5 Host neuroprotection by human NPCs (Paper IV)

In both contusion and compression SCI models, the hindlimb motor function of rats was significantly improved by SC-NPCs transplanted subacutely. The effect of acutely transplanted SC-NPCs was even more pronounced in the compression injury compared to the contusion injury model, reaching a difference between transplanted and non-transplanted groups of more than 4 points of the Basso, Beattie, Bresnahan locomotor rating scale score. In contrast, transplantation at the chronic stage was without beneficial effect. A lack of treatment effect was also seen in animals transplanted subacutely with FBr-NPCs, which contrasts to a previous report with observed similar functional improvement with rodent SC-NPCs and FBr-NPCs in a SCI contusion model (Watanabe *et al.* 2004).

The therapeutic effect of SC-NPCs declined when the delay between injury and transplantation increased, suggesting that neuroprotection is an important mechanism of the grafted NPCs to rescue dying neurons in the injured spinal cord. All SCI rats suffered a major loss of neurons in the injured part of the cord. In the acutely grafted rats showing the best functional outcome, the number of surviving host neurons in the injured spinal cord was almost 3 times as many as in non-transplanted rats. In the hosts with subacute transplantation, the second best functional outcome was paralleled by twice as many surviving host neurons as in the control group. The other groups, which did not achieve better functionality than the control group, also did not contain more surviving host neurons than the control group. This strongly indicated that

neuroprotection is one of the main therapeutic effects of the grafted NPCs. The cell type(s) responsible for the protective effect are most likely the immature NPCs, which mainly are present during the 7–9 days therapeutic window, while differentiation takes place weeks-months after grafting. Accordingly, the ratios of differentiated cell types were not correlated to functional improvement.

The common experimental design studying transplantation of human cells constitutes a xenotransplantation, in contrast to the allogeneic situation in clinic. Even if graft rejection can be avoided (as in the clinic) by using immunosuppressant or here experimentally by utilizing genetically immunodeficient animals, the resulting immunemodulation may interfere with mechanisms studied. To address this, a contusion injury model in a human organotypic spinal cord slice culture system was used to offer an allotransplantation-like situation. The analysis of active caspase-3-positive host cells, in the injured human fetal spinal cord slices, showed that human NPC transplantation prevented the caspase-3-dependent apoptosis, demonstrating a strong protective effect.

5 CONCLUSIONS

The overall aim of this thesis project was to improve the understanding of host responses and interactions between host and donor cells in human neural cell therapy after SCI.

The immunocompetence, immunogenicity and interactions of two potential human NPCs with human lymphocytes were studied. hESC-NPCs, but not hfNPCs, triggered an allogeneic response in a dose-dependent manner. This suggests that in the naïve non-stimulated encounter between incompatible donor NPCs and host lymphocytes, hESC-NPCs are more immunogenic than hfNPCs. Despite the fact that hESC-NPCs trigger lymphocyte proliferation, neither hESC-NPCs nor hfNPCs enhanced an established lymphocyte alloresponse. In contrast, both types of human NPCs reduced this proliferative response. Our findings indicate that both human fetal and ESC-derived NPCs have immunomodulatory effects, and can counteract an activated ongoing host alloreaction.

Both types of human NPCs increased the pool of regulatory T cells, when in direct cell-cell contact during cultures. However, non-stimulated hfNPCs (without presence of the cytokines TNF-α or IFN-γ) secreted more TGF-β than hESC-NPCs. Furthermore, there was an increased level of TGF-β1 in the media and present in the cells in co-cultures of lymphocytes and hfNPCs compared to co-cultures with hESC-NPCs. Lymphocyte proliferation could be reduced by exogenous addition of TGF-β. In addition, TGF-β1 antibody treatment resulted in a higher lymphocyte proliferative response in co-cultures with hfNPCs. Taken together, these results suggest that TGF-β1 induced by hfNPCs may exert suppressive effects, possibly via an induced $FOXP3^{+}$ regulatory T cell population. We conclude that human NPCs of different origin display similarities but also significant differences in their immunocompetence and interaction with allogeneic lymphocytes. These differences may affect a host response, and potentially the outcome of neural cell therapy.

With the aim to mimic a naïve encounter of donor human NPCs with allogeneic microglia, we performed another set of co-cultures. In the presence of hfNPCs, the proliferation and phagocytic activity of human microglia were increased, as well as the proportion of microglia positive for M1-marker HLA-II or M2-marker CD206. These

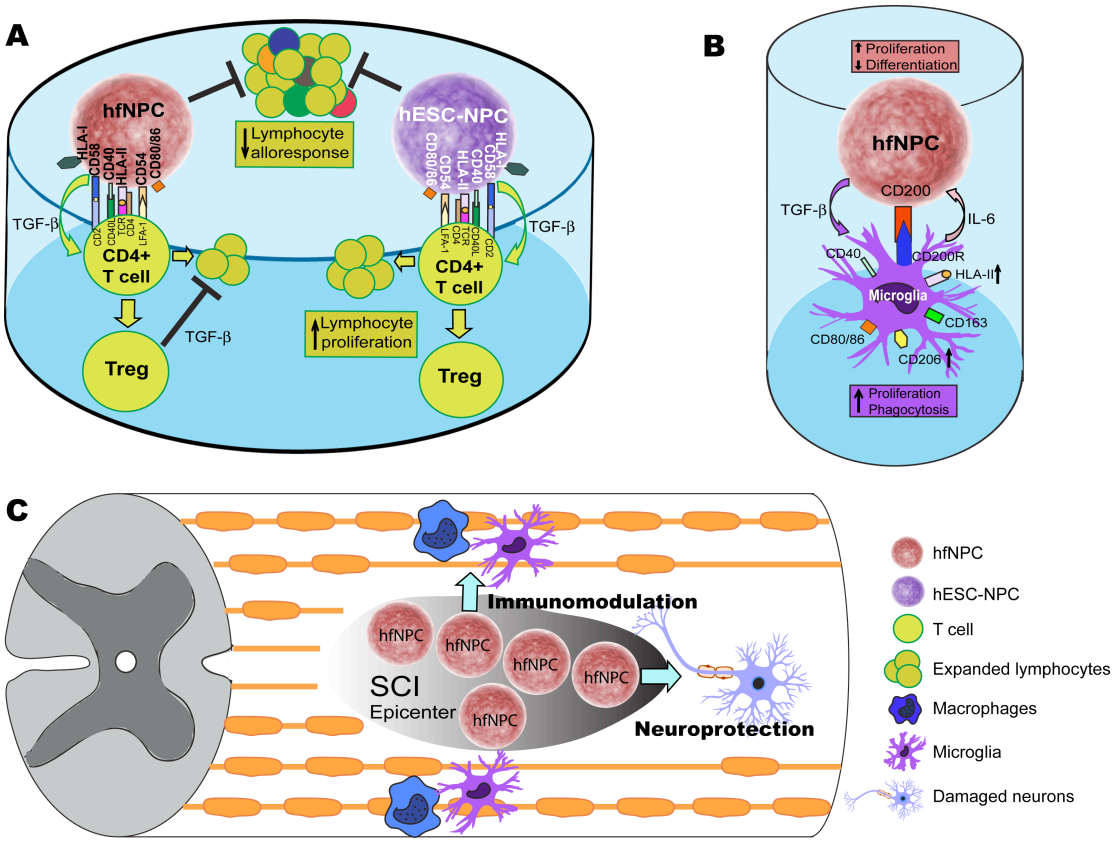

Fig.2. A schematic summary and my hypothesis concerning human NPC and host cell interactions of relevance for neural cell therapy in SCI. (A) The immunogenicity of hESC-NPCs and hfNPCs and their interactions with human lymphocytes *in vitro*. Both hESC-NPCs and hfNPCs expressed HLA, co-stimulatory and/or adhesion molecules, and increased the pool of Tregs. hESC-NPCs, but not hfNPCs, triggered allogeneic lymphocyte proliferation, but both types of human NPCs reduced an ongoing lymphocyte alloresponse. TGF-β1 induced by hfNPCs may exert suppressive effects, possibly via induced $FOXP3^{+}$ Tregs. (B) Interplay between hfNPCs and human microglia *in vitro*. The presence of hfNPCs increased the proliferation, phagocytosis of human microglia, as well as the proportion of $HLA\text{-}II^{+}$ or $CD206^{+}$ microglia. Co-culture with microglia increased the proliferation of hfNPCs, but hindered their differentiation. The CD200-CD200R interaction was enhanced in the co-culture of hfNPCs and human microglia. (C) The immunomodulatory and neuroprotective effects of hfNPCs *in vivo*. Transplanted hfNPCs in the injured spinal cord presented an immunomodulatory effect by decreasing post-lesion macrophage/microglial activation. Finally, hfNPCs exerted a neuroprotective effect by enhancing the survival of the host neural cells in the injured spinal cord.

data suggest that transplanted hfNPCs can influence the activation and functional activity of host microglia, and that different microglial phenotype can emerge. Co-culture with microglia also increased the proliferation of hfNPCs but hindered their differentiation with regard to both neuronal or astroglial fate. Thus, the interplay

between human NPCs and microglia significantly affected their respective proliferation and phenotype. Furthermore, in *in vivo* studies on hfNPC xenotransplantation, host microglial/macrophage ED1 immunoreactivity was significantly decreased at the lesion epicenter after a moderate injury compared to non-grafted cases. This finding was confirmed in a human allogeneic setting by employing an *in vitro* human organotypic spinal cord slice culture model. From our *in vitro* and *in vivo* studies we conclude that cell therapy using human NPCs as donor, in addition to cell replacement, also can reduce the host microglial response to SCI.

Finally, hfNPC xenotransplantation was performed in both contusion and compression SCI in rodents. In both SCI models, human spinal cord-derived NPC transplantation within 9 days after lesion significantly improved host functional outcome. The improved function correlated with an increase of host neurons in the spinal cord. A neuroprotective effect of human NPCs was further confirmed in a human allogeneic setting using human organotypic spinal cord slice cultures. These results from multiple SCI models as well as in two different species, including human tissue, indicate a robust neuroprotective effect by human donor NPCs in SCI.

Increased understanding of the immunocompetence and immunomodulatory effects, as well as the neuroprotective potential of transplanted neural cells is of significant importance since it can improve matching of donor and host, the choice of time point for intervention and the design of immunosuppressive treatments for clinical neural cell therapy in SCI.

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